AIX-MARSEILLE UNIVERSITÉ

FACULTÉ DE MÉDECINE DE MARSEILLE

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THÈSE

DÉTECTION DES MICROORGANISMES À PARTIR DE LA PULPE DENTAIRE ANCIENNE

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AVANT PROPOS

Le format de présentation de cette Thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille.

Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de Thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les Thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette Thèse. Par ailleurs, la Thèse présentée sur article publié, accepté ou soumis associé d'un bref commentaire donne le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Pr. Didier Raoult

SOMMAIRE

RÉSUMÉ

La revue de la littérature montre que la pulpe dentaire est une source utile pour le diagnostic des bactériémies, y compris en paléomicrobiologie. Les précédents travaux en paléomicrobiologie réalisés dans le laboratoire ont tous mis en évidence une amplification possible de courts ou très courts fragments d'ADN bactérien partant de l'hypothèse que l'ADN ancien est fragmenté. Ces observations nous ont amenés à essayer de mieux comprendre ces phénomènes de dégradation de l'ADN ancien dans le temps au cours du premier travail de cette thèse. Dans ce premier travail, le modèle expérimental de dégradation de l'ADN des macrophages murins J774 et de *Mycobacterium smegmatis* par la chaleur sèche à 90°C a montré une différence statistiquement significative ($p < 0.05$) entre la vitesse de fragmentation de l'ADN bactérien et eucaryote. En fait, les PCRs en temps réel ont amplifié les fragments de 750-paires de bases (-pb) de l'ADN de *M. smegmatis* après 48 heures d'incubation à 90°C tandis que seulement les fragments de 450-pb de l'ADN des macrophages ont été amplifiés dans les mêmes conditions. Le modèle des bactéries intracellulaires, macrophages infectés par *M. smegmatis*, a également montré que l'ADN des mycobactéries était plus résistant que l'ADN eucaryote. Ces résultats suggèrent que les diagnostics paléomicrobiologiques peuvent détecter des fragments plus longs d'ADN bactérien à partir des échantillons anciens. Dans un deuxième travail, un système de détection rapide de 7 agents pathogènes par PCR multiplex en temps réel a été utilisé pour détecter ces pathogènes suspectés à partir de la pulpe dentaire de 1192 dents anciennes collectées dans 12 charniers dont un localisé à Douai (1710 – 1712). Après la détection de *Bartonella quintana* dans ce charnier, les PCRs en temps réel emboîtées ultra-sensibles et la «PCR suicide» ont été utilisées pour confirmer la présence de *Rickettsia prowazekii* souche Madrid E génotype B

dans 6/55 pulpes dentaires (11%) collectées de 6/21 squelettes (28.6%) de soldats à Douai. Ces résultats supportent l'hypothèse que le typhus a été introduit en Europe par les soldats espagnols au retour des conquêtes en Amérique. Dans un dernier travail, l'ADN extrait à partir de la pulpe dentaire de 5 dents datées du 17ème au 18ème siècle et collectées dans le charnier d'Issoudun, a été analysé par pyroséquençage massif. Le pyroséquençage a permis de détecter des séquences de *Yersinia pestis*. Ces résultats ont été confirmés ultérieurement par les PCRs et le génotypage de *Y. pestis*. Il s'agissait du génotype Orientalis. Cette détection a été inattendue parce que la cause du décès de ces individus à Issoudun n'était pas encore identifiée malgré plusieurs tentatives de détection moléculaire et l'hypothèse d'une peste avait été écartée par les anthropologues. Pour son application dans la paléomicrobiologie, le pyroséquençage massif représente vraiment une technique hautement sensible qui peut être utilisée comme première tentative pour la détection de tous les pathogènes suspectés et autres à partir des échantillons anciens. Nous avons appelé cette nouvelle approche «paléométagénomique».

Mots clés : pulpe dentaire, dent ancienne, paléomicrobiologie, détection moléculaire, dégradation de l'ADN, paléométagénomique

SUMMARY

Reviewing the literature shows that dental pulp is a useful source for bacteremic and paleomicrobiological diagnoses. In the first work, an experimental model of DNA degradation of the murine macrophage cell line J774 and *Mycobacterium smegmatis* by exposure to 90°C dry heat showed a statistically significant difference ($p < 0.05$) of fragmentation level between bacterial and eukaryotic DNA. Indeed, real-time PCR amplified of 750-base pairs (-bp) *M. smegmatis* DNA after 48-hour 90°C incubation whereas only 450-bp macrophage DNA was amplified in the same condition. Experimental model of intracellular bacteria of *M. smegmatis*-infected macrophages also showed that mycobacterial DNA was more resistant to dry-heat stress than eukaryotic DNA. These results suggest that paleomicrobiological diagnosis can detect more large fragments of bacterial DNA from ancient buried specimens. In the second work, a system of rapid detection of seven pathogens by multiplex real-time PCR was used for detecting suspected pathogens from dental pulp of 1192 ancient teeth collected from 12 multiple burials including a mass grave in Douai, 1710 – 1712. After the *Bartonella quintana* detection in this site, real-time nested PCR and ultra-sensitive "suicide PCR" were used to confirm the presence of *Rickettsia prowazekii* strain Madrid E genotype B in 6/55 dental pulp specimens collected from 6/21 (29%) skeletons of soldiers buried in Douai.These results support the hypothesis that typhus was imported into Europe by Spanish soldiers from America. In the last work, DNA extracted from 5 dental pulp specimens collected from multiple burials at Issoudun, 17^{th} – 18^{th} centuries, was analyzed by pyrosequencing which detected *Yersinia pestis* sequences in the metagenome. These results were ultimately confirmed by PCR and genotyping of *Y. pestis* Orientalis strain. This detection was unexpected because previous tests of infectious diseases

including plague were all negatives and therefore the cause of these deaths was not identified yet. For paleomicrobiology, pyrosequencing is a sensitive technic which can be used as baseline test to detect both suspected and unexpected pathogens from ancient specimens. We named this new approach «paleometagenomics».

Key works: dental pulp, ancient tooth, paleomicrobiology, molecular detection, DNA degradation, paleometagenomics

INTRODUCTION & OBJECTIFS

La pulpe dentaire est un tissu conjonctif spécialisé qui occupe une position centrale au sein de la dent. Les bactéries qui circulent dans le sang peuvent coloniser la pulpe dentaire. Un modèle expérimental d'un animal bactériémique a montré que la pulpe dentaire pouvait être utilisée pour diagnostiquer des bactériémies (Aboudharam et al. 2000, Aboudharam et al. 2004a, Aboudharam et al. 2004b). Par ailleurs, la pulpe dentaire est le seul tissu conjonctif persistant plusieurs milliers d'années après la dégradation des autres tissus mous car elle est très préservée à l'intérieur de la dent, organe le plus dur du corps humain. Puisque le protocole d'extraction de l'ADN à partir d'un tissu organique est plus simple que celui utilisé pour extraire des acides nucléiques de l'os, la pulpe dentaire ancienne a été proposée comme un outil fiable pour les diagnostics paléomicrobiologiques (Drancourt & Raoult 2005). En fait, après la détection princeps de l'ADN de *Yersinia pestis* dans la pulpe dentaire collectée à partir des dents anciennes à Marseille, 1720 – 1722 (Drancourt et al. 1998), la cause de la Peste Noire qui a tué un tiers de la population européenne du 14ème au 18ème siècle a été élucidée (Drancourt 2012, Raoult et al. 2000). La détection de l'ADN de *Y. pestis* dans la pulpe dentaire (37%) est plus élevée que dans l'os (5.7%) et la détection de la peste ancienne est également plus efficace à partir de la pulpe dentaire (20%) par rapport à l'os (14%) (Drancourt & Raoult 2011). Donc, l'utilisation de la pulpe dentaire ancienne est actuellement acceptée comme une clé de succès pour la recherche de *Y. pestis* ancien ainsi que les études paléomicrobiologiques.

La «PCR suicide» et le génotypage à partir des espaces intergéniques ont été proposés par le laboratoire comme les méthodes de référence pour la paléomicrobiologie (Drancourt et al. 2004, Drancourt & Raoult 2005, Raoult et al. 2000). Pourtant, ces méthodes sont limitées par l'identification d'un seul pathogène suspecté à partir d'échantillons anciens. C'est pourquoi

une nouvelle méthode de détection rapide de plusieurs pathogènes simultanément, la PCR multiplex en temps réel par exemple, est utile. Deux études publiées en 2011 ont combiné séquençage massif et enrichissement de l'ADN de *Y. pestis* à partir de la pulpe dentaire ancienne pour reconstruire le génome ancien de *Y. pestis* (Bos et al. 2011, Schuenemann et al. 2011). Cette nouvelle technologie, pyroséquençage, peut probablement être utilisée pour détecter en aveugle tous les pathogènes suspectés et autres dans la pulpe dentaire ancienne.

Les études des microorganismes à partir des échantillons anciens ont suggéré que la paroi épaisse, l'enkystement et le mécanisme de réparation de l'ADN endommagé chez les mycobactéries pouvaient les protéger de la dégradation de l'ADN. Alors, une hypothèse a été proposée: l'ADN bactérien est plus résistant que l'ADN eucaryote (Donoghue et al. 2004, Zink et al. 2002). Puisque les recherches paléomicrobiologiques sont souvent basées sur les connaissances de paléobiologie qui a confirmé la dégradation de l'ADN ancien en petits fragments de 100 à 200 paires de bases (Paabo et al. 2004), la mise en évidence de cette hypothèse pourra ouvrir des nouvelles stratégies de recherche pour la paléomicrobiologie.

Les objectifs du travail de cette Thèse ont été :

1. La mise en évidence de l'hypothèse selon laquelle «l'ADN bactérien est plus résistant que l'ADN eucaryote» par l'étude d'un modèle expérimental et ses applications en paléomicrobiologie.

2. Le développement d'une nouvelle méthode, la PCR multiplex en temps réel, pour détecter rapidement et simultanément plusieurs pathogènes suspectés d'infections à partir de la pulpe dentaire ancienne.

3. Le développement d'une nouvelle approche que nous avons appelé la paléométagénomique pour détecter tous les microorganismes présent dans la pulpe dentaire ancienne.

Chapitre I

Mini review : Dental pulp as a source for paleomicrobiology

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Chapitre I – Avant propos

La paléomicrobiologie est une science qui étudie les microorganismes à partir des échantillons anciens. La dent est l'organe le plus dur de l'organisme humain et la pulpe dentaire est un tissu conjonctif très préservé et protégé à l'intérieur de la cavité pulpaire. Les bactéries circulant dans le sang peuvent coloniser la pulpe dentaire; elle a donc été proposée comme un tissu utile pour diagnostiquer des bactériémies anciennes. L'application de l'utilisation de la pulpe dentaire dans la paléomicrobiologie pour identifier de l'ADN bactérien à partir des dents anciennes a été basée principalement sur la «PCR suicide» et typage des espaces intergéniques. Le succès de détection de *Yersinia pestis*, *Bartonella quintana*, *Rickettsia prowazekii*, *Salmonella enterica* serovar Typhi, *Mycobacterium tuberculosis* and *Mycobacterium leprae* à partir de la pulpe dentaire ancienne a contribué à établir l'étiologie de certaines épidémies passées.

Mini Review: Dental Pulp as a Source for Paleomicrobiology

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Abstract

Paleomicrobiology is a recent science. Paleomicrobiology describes the history of infectious diseases through research of ancient microbes. The dentistry with the tooth in particular has contributed to the progress of this science. The dental pulp is very well protected in the centre of the tooth. Molecular techniques such as “suicide PCR” and Multiple Spacer Typing have identified and characterized micro-organisms in the ancient samples. The detection of bacterial DNA from ancient dental pulp provided the scientific evidences for diagnosis of the infectious diseases of the past. The cooperation of paleomicrobiology and paleodontology also contributed the new knowledge to human pathology.

Keywords: Paleomicrobiology; Ancient Teeth; Dental Pulp; Microbial Detection

Introduction

In 1993, after the first molecular detection of Mycobacterium tuberculosis in ancient skeletons (1), paleomicrobiology appeared as a new discipline for the identification and characterization of microorganisms in the ancient samples. Frozen tissues, mummies, skeletons or bones were the ancient remains often used in paleomicrobiological studies. However, the major limitations of the ancient DNA (aDNA) study consist in the degradation and fragmentation of aDNA, contamination of modern DNA or external sources. Moreover, aDNA extraction from mummified tissue and bones is complicated because rehydration and decalcification are necessary steps. Therefore, another source of ancient specimens that possibly limited the disadvantages above, were proposed: dental pulp (2), Figure 1. Vascularization of this organ is important, proportionally comparable with the human brain (3). Pathogenic bacteria circulating in blood may occur and colonize the dental pulp. A guinea-pig

model has confirmed the possibility to detect bacterial DNA in dental pulp after experimental infection by Coxiella burnetii. This experimental model has also confirmed that pathogenic bacteria in blood had penetrated and colonized the dental pulp and that could then be used for diagnosis of bacteremia (4,5). Moreover, Bartonella henselae DNA and Bartonella grahamii DNA had been isolated from the dental pulp of the cats that had been bacteremic for Bartonella sp. then euthenased and buried one year previously, according to French army veterinary records (6). These results indicate that dental pulp was an excellent specimen for diagnosis of ancient bacteremia.

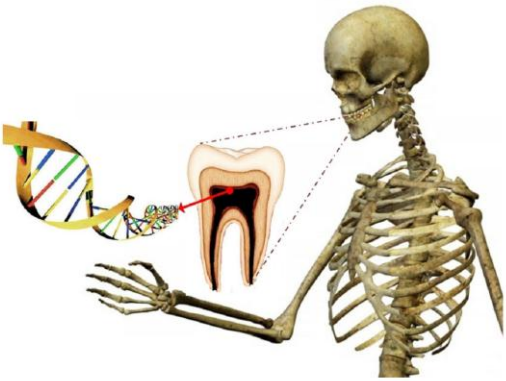

Figure 1 Ancient DNA recovered from dental pulp.

Paleomicrobiology of the dental pulp: methods

In 1998, a team of researchers in France had first introduced dental pulp collected from ancient teeth as a suitable tool for the diagnosis of ancient infectious diseases (7). The advantages of using dental pulp for paleomicrobiological study is its better preservation in a closed cavity. In the closed cavity, the soft tissues are preserved from environmental contamination and attacks of degrading agents. Moreover, the DNA extraction from soft tissues of the dental pulp is simple (2). An experimental animal model infected with Coxiella burnetii had also confirmed the possibility of finding bacterial DNA in dental pulp 20 days after bacteremia (4). A second guinea-pig model infected with Coxiella burnetii showed persistence of the bacteria in the dental pulp (5). The pulp can then be used as a tool for retrospective diagnosis when there was a bacteremia. Afterwards, this team proposed the "suicide PCR" for molecular identification of bacterial DNA from ancient teeth with strict criteria to avoid any risk of contamination and Multiple Spacer Typing for the genotyping of ancient bacteria (8). The important criteria of "suicide PCR" included: 1- The primers are used only once, 2-There were no positive controls, 3-The negative controls should be added, 4-Negative results are tested by other primers, 5-Positive results are confirmed by sequencing (9). The suicide PCR principle was used by another Greek team in 2006. This team using dental pulp has shown that the plague of Athens was in

fact typhoid fever (10). Rapid Diagnostic Test was an immunodetection method developed by another team for special diagnosis of plague from ancient remains (11). From 1998 to the present day, several independent teams using these methods, detected multiple pathogens from ancient dental pulp to confirm the epidemics of the past, Table 1.

Table 1 Microorganisms detected from ancient teeth

Microorganisms	Infectious diseases	References
Anelloviridae	Virus	25
Bartonella henselae	Cat-scratch disease	24
Bartonella quintana	Trench fever	17-19;21;22
Mycobacterium leprae	Leprosy	23
Mycobacterium tuberculosis	Tuberculosis	23
Rickettsia prowazekii	Typhus	21;22
Samonella enterica serovar Typhi	Typhoid fever	10
Yersinia pestis	Plague	7-9;11;13-18

Paleomicrobiology of the dental pulp: contributions

In history, the three plague pandemics were cited as humanity's disaster, in which an epidemic known as Black Death occurred in 1347 and then spread from the Caspian Sea to almost all European countries. Over the next few years, Black Death had killed an estimated 17 – 28 million Europeans, representing approximately one third of the population at the time (12). In 1998, Yersinia pestis DNA had first been identified in dental pulp of the ancient teeth collected from skeletons excavated from 16th and 18th century graves of persons suspected of having died of plague (7). Genotyping had then yielded Y. pestis Orientalis strain (8,13). These results were later reinforced by other independent teams to confirm the presence of plague in Medieval Europe (11,14-16). Interestingly, two recent studies detected co-infection of the body louse, Bartonella quintana, along with Yersinia pestis in ancient dental pulp, suggesting that the body louse may have been a vector for interhuman transmission of medieval plague pandemics (17-18).

Bartonella quintana is the causative agent of trench fever disease and transmitted by lice, fleas and ticks to humans. Molecular detection of B. quintana in a 4000-year-old human tooth (southeastern France) indicated that B. quintana bacteremia had occurred in prehistoric humans 19. Typhus epidemic had been demonstrated in the 20th century as "a best friend" of wars. In the past, this epidemic spread rapidly in Europe from Spain to France and Italy following the wars between Emperor Charles V and King François I (20). The city of Douai in northern France was besieged from 1710 to 1712 during the war of Spanish succession when France and Spain opposed the other nations. The causative agent of typhus, Rickettsia prowazekii strain Madrid E type B, and B. quintana DNA had been isolated from ancient dental pulp of the individuals buried in Douai. This molecular detection

allowed the researchers to confirm the presence of the typhus epidemic in Douai from 1710 – 1712 (21). Typhus was later a constant companion of Napoleon's army in all European wars. In 2006, evidence of louse-transmitted diseases in soldiers of Napoleon's Grand Army in Vilnius was reported when R. prowazekii and B. quintana DNA had been amplified by "suicide PCR" from dental pulp collected from the remains of the soldiers. These results showed that louse-borne infectious diseases had affected nearly one-third of Napoleon's soldiers buried in Vilnius and suggested a possible cause of the French retreat from Russia (22), Figure 2 and 3.

Mycobacterium tuberculosis and Mycobacterium leprae had also been detected in ancient teeth recovered from a 1st century tomb in Jerusalem, Israel. The findings had suggested the co-infection of these two mycobacteria in ancient human populations (23). Moreover DNA of Samonella enterica serovar Typhi had been successfully identified in dental pulp of ancient teeth collected from Kerameikos cemetery. This discovery had demonstrated that typhoid fever could be the cause of the plague of Athens in 430 – 426 BC (10). Interestingly, molecular detection of Bartonella henselae in dental pulp of 800-year-old cats suggested the occurrence of Cat-scratch disease within a medieval population in France because archaeological and historical data indicated that these cats had lived in close contact with people (24). A recent study had surprisingly reported the detection of an ancient virus (Anelloviridae DNA) from 200-year-old dental pulp recovered from ancient teeth of Napoleon's soldiers in Kaliningrad, Russia (25).

Figure 2 and 3 Vilnius, 1812, Pictures of Yann ARDAGNA (UMR 6578 CNRS Université de la Méditerranée-CNRS-EFS

Perspectives

X-rays were used to detect and locate the target teeth in the jaws (Figures 4 and 5). The ancient tooth was usually bisected longitudinally for the recovery of dental pulp (Figures 6, 7 and 8). This method led to the destruction of its morphological structure and possible external contamination . "Orthograde entrance technique" is an "original method" to collect dental pulp with morphological conservation of ancient teeth that will be used afterwards by anthropologists (26). However, this technique has some disadvantages due to the relatively fragile, permeable and weak nature of the tooth root. The first method is probably the simplest and most effective despite the disadvantage of morphological changes of the tooth, it is necessary to restore the tooth by bonding techniques.

The ancient proteins seem to be more resistant than aDNA. Lipids such as mycolic acids of M. tuberculosis also appear to be particularly robust in ancient remains (27). Mass spectrometry had successfully identified specific bacterial proteins of M. tuberculosis from archeological bone samples (28). Therefore, this method, detecting microbial non-nucleotidic biomolecules from ancient dental pulp, may hereafter become a promising approach to complement aDNA analysis in paleomicrobiology.

In conclusion, detection of pathogen traces from ancient teeth by using many paleomicrobiological methods has provided the scientific proofs to elucidate several infectious diseases in the past. This great cooperation of paleomicrobiology and paleodontology has also contributed new knowledge of human pathology as well as evidence of human history.

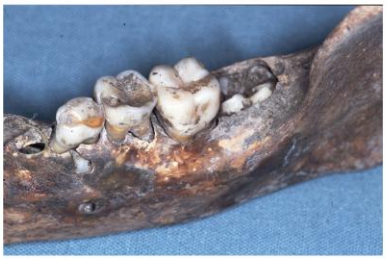

Figure 4 Mandible of a child about 9 years (Observance massgrave-Marseille 1724)

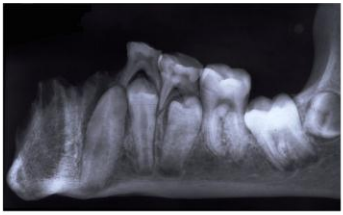

Figure 5 Radiography of the mandible and choice of the tooth (34 non-eruptive)

Figure 6 Realization of the fracture using a carborundum disc on handpiece

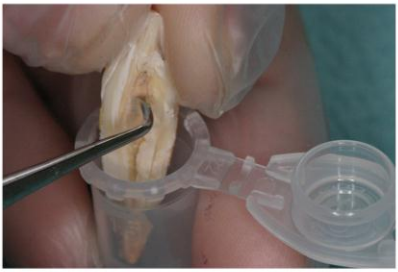

Figure 7 Recovery of the dental pulp

Figure 8 After the fracture and recovery of the pulp, tooth is ready to be glued

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Chapitre II

Dental pulp microbiota in intact chamber

Tung Nguyen-Hieu, Michel Drancourt, Gérard Aboudharam

Writting

Chapitre II – Avant propos

L'analyse microbiologique de la pulpe dentaire est souvent réalisée à partir de dents présentant des lésions carieuses et/ou des infections parodontales. L'objectif de cette revue est d'établir un répertoire exhaustif des microorganismes de la pulpe dentaire des dents humaines anciennes et contemporaines ayant une chambre pulpaire indemne. Les études expérimentales ont démontré que les bactéries pouvaient coloniser la pulpe dentaire pendant une bactériémie. La détection moléculaire des pathogènes causant des maladies infectieuses dans le passé comme *Yersinia pestis, Rickettsia prowazekii, Bartonella quintana, Mycobacterium tuberculosis, Mycobacterium leprae* and *Salmonella enterica* a confirmé l'infection de la pulpe dentaire causée par les bactéries provenant du sang. A partir des dents anciennes et modernes de l'homme, 49 genres bactériens avec les champignons et les virus ont été détectés dans la pulpe dentaire prélevée à partir de chambres pulpaires macroscopiquement indemnes. Cette revue a révélé une population microbienne colonisant initialement la pulpe dentaire comme des microorganismes potentiellement responsables d'infections pulpaires primaires. Les études ultérieures sont nécessaires pour compléter ce répertoire en particulier avec les archaea et les virus.

Dental pulp microbiota in intact chamber

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Abstract

Microbiological analyses of the dental pulp were usually performed from teeth with oral communications via carious lesions and periodontal infections. The aim of this review was to establish the repertoire of dental pulp microorganisms in ancient and modern human teeth with an intact pulp chamber. Experimental studies demonstrated that bacteria could colonize the dental pulp during bacteremia. Molecular detection of the causative agents of some past infectious diseases, including *Yersinia pestis, Rickettsia prowazekii, Bartonella quintana, Mycobacterium tuberculosis, Mycobacterium leprae* and *Salmonella enterica*, confirmed blood-borne infection of the dental pulp. In both modern and ancient teeth, a total of 49 bacterial genera along with yeasts and viruses were detected from human dental pulp specimens collected from macroscopically intact chamber. This review therefore revealed a microbial population initially colonizing the dental pulp as microorganisms potentially responsible for primary pulpal infections. Further studies are required to complete this repertoire with particularly attention to archaea and viruses.

Keywords: microbiota, human teeth, ancient teeth, dental pulp, intact pulp chamber, intact teeth

Contents

1. Introduction

The natural sterility of the dental pulp remains a controversial issue. While 15% of traumatized teeth with intact crowns presenting pulp necrosis were found to be sterile,[1] no recent publication reports on healthy dental pulp microbiota in the intact teeth. Therefore, it has been hypothesed that the dental pulp was naturally sterile[2] and that pulpal infection develops only after pulp necrosis and the loss of the dental pulp defenses[3] during anachoresis, a process by which microbes may be transported by the blood or lymph to inflammatory area where they establish an infection.[4] Microbiological studies performed from diseased pulp specimens indicated that bacteria, fungi, archaea and viruses were found in asymptomatic and symptomatic primary infections as well as in refractory and persistent infections after an endodontic treatment.[3,5] However, the majority of these studies and reviews reported the microbiota of the pulp chamber exposed to the oral environment via carious lesions, fistula and periodontal pockets (Figure 1), whereas no review summarized microorganisms found in the intact pulp chamber.

2. Blood-borne infection of the dental pulp: experimental models

Studying the colonization of microorganisms in inflammed dental pulps following bacteremia was carried-out by injecting *Staphylococcus aureus* into the femoral vein of Wistar rats.[6] After 24-hour bacteremia, heart blood samples and dental pulp specimens collected from untreated control teeth were cultured. *S. aureus* was detected in 18/153 (12%) and 3/128 (2%) dental pulp specimens of *S. aureus*-positive and *S. aureus*-negative heart blood rats, respectively. After 7-day bacteremia, *S. aureus* was detected only in inflammed dental pulps which were previously exposed by cavity preparation and treated with croton oil, cement and eugenol-zinc oxide.[6] Dogs were intravenously inoculated with bacteria including Gram-positive

cocci, *Escherichia coli* and beta-hemolytic streptococcus.[7] Inflammation of the dental pulp was induced by preparing deep, clinically unexposed class V cavity which were left open or treated with croton oil and eugenol-zinc oxide. Bacteria were not detected in uninflammed dental pulps of the control teeth whereas they were identified in the inflammed dental pulps by culture associated with fluorescent antibody detection.[7] In another experimental model of bacterial anachoresis, dog dental pulps were previously treated with Dycan, calcium hydroxide and Teflon.[8] After 24-hour streptococci bacteremia, bacteria were observed only in the inflammed dental pulp of treated teeth but they were not found in the pulps of untreated control teeth. These findings supported the conjecture that during bacteremia, the inflammatory reactions attracted bacteria to the dental pulp.[8] Studying guinea-pigs experimentally infected by the intracellular bacterium *Coxiella burnetii* further established that the dental pulp could be infected by blood-borne bacteria.[9,10] Bacterial DNA and viable bacteria were detected and cultured in the dental pulp of challenged bacteremic animals and not in the negative controls. However, bacteria were not present in all dental pulp specimens of bacteremic animals, supporting the hypothesis that some local factors could be involved in blood-borne infection of the dental pulp. *Bartonella quintana* and *Bartonella henselae*, the respective agents of trench fever and cat-cratch disease in humans, were detected from teeth of 2/9 (23%) domectic cats.[11] These results are in agreement with observation of patients having close contacts with cats and diagnosed with *B. quintana* chronic infection.[12] A further study of *Bartonella* bacteremic cats buried for one year, found *B. henselae* and *Bartonella grahamii* DNA in the dental pulp, indicating that buried dental pulp was a suitable specimen of which to diagnose past bacteremia.[13,14] An experimental guinea-pig model of scrapie (Creutzfeldt-Jakob disease or prion disease) investigated potential route of infection in dental procedures.[15] After intraperitoneal inoculation of the

scrapie agent, infectiousness was found both in the nervous system as well as in the dental pulp of challenged animals. Direct injection of prion into the pulp cavity also brought scrapie disease in the guinea-pigs, suggesting that the scrapie agent could spread from the dental pulp to the central nervous system and also in the inverse way.[15]

3. Dental pulp microorganisms identified from skeletons

Dental pulp collected from ancient teeth was used as a specimen suitable for paleomicrobiological researches.[16] Advantages of dental pulp in paleomicrobiological studies comprise the long persistence of the dental pulp in the tooth that is the hardest organ of the human body, its preservation in a "closed" cavity that limits environmental contamination as well as attacks of degradative agents and ultimately a simple DNA extraction from soft tissues of the pulp.[14,17] Indeed, bacteremic infectious diseases such as plague,[16,18-27] typhus,[28,29] trench fever[25,26,28-30] and typhoid fever[31] were diagnosed by using the ancient dental pulp specimens and "suicide PCR"[23] (Supplementary table 1). Multiple spacer typing successfully identified *Yersinia pestis* Orientalis-like strain that caused two historical plague pandemics.[20] Two recent studies using DNA enrichment coupled with high-throughput sequencing permitted the reconstruction of 93% of the *Y. pestis* genome from ancient dental pulp specimens collected from victims of the Black Death in London (1348 – 1350).[24,32] Moreover, DNA of *Salmonella enterica* serovar Typhi has been amplified from ancient teeth collected from Kerameikos cemetery, demonstrating that typhoid fever was the cause of the plague of Athens in 430 – 426 BC.[31] *B. quintana*, the causative agent of trench fever disease, is transmitted by lice to humans[30] as is *Rickettsia prowazekii*, the causative agent of epidemic typhus. An evidence of louse-transmitted diseases in soldiers of Napoleon's Grand Army was reported when PCR and sequencing revealed both *B. quintana*

and *R. prowazekii* DNA from dental pulp specimens collected from buried skeletons at Vilnius.[29] Likewise, molecular detection allowed researchers to confirm the presence of epidemic typhus in Douai from 1710 – 1712 during the war of Spainish succession.[28] *Mycobacterium tuberculosis* and *Mycobacterium leprae* were also detected in dental pulp of ancient teeth collected from 1st century tomb discovered in Jerusalem.[33] Surprisingly, *Anelloviridae* DNA was amplified by PCR from 200-year-old dental pulp specimens recovered from ancient teeth of soldiers of Napoleon's Grand Army in Kaliningrad.[34] Although *Anelloviridae* viruses are found with > 95% prevalence in the blood of modern individuals,[35] the successful detection of such an ubiquitous, non-integrated, DNA virus from ancient dental pulp[34] suggested the possibility of investigating viruses from ancient biological materials. Overvall, the use of PCR-based methods, pyrosequencing[24,32] and immunodetection[19] detected a total of six causative agents of infectious diseases from the ancient dental pulp (Figure 2).

4. Dental pulp microorganisms identified in patients

4.1 Appreciation of dental pulp chamber integrity

In all papers herein reviewed, verification of tooth integrity was only based on the visual observation of clinicians and radiography. Therefore, all large carious lesions, crown fractures/cracks, abscesses/sinus tracts and deep periodontal pockets were readily detected whereas some early caries, tooth wears or cementum loss at cervical area leading to oral exposure of dentinal tubuli could be not excluded. Moreover, intact teeth might have enamel micro-cracks[36] which were possible access of oral microorganisms.[37] However, establishing microbiota of such macroscopically intact teeth could reveal microbial population initially colinizing the dental pulp.

4.2 Bacteria

4.2.1 Dental pulp specimens collected from extracted teeth

Sixty-four apparently intact teeth were extracted and then treated with antiseptics.[38] Of which, thirty teeth externally sterile at the end of 8 days' culture were used for bacteriological and histological investigations of the dental pulp. *Streptococcus viridans,* was isolated from 10/30 dental pulp specimens. Histologically, *Streptococcus* ssp. may be found in the pulp of intact teeth without any inflammatory signs indicated by leucocyte infiltration.[38] However, bacteria were present generally in clumps in a circumscribed area, suggesting their multiplication in the pulp during the 8 days' incubation of the tooth. Moreover, pressure or suction during tooth extraction, particularly in luxation of the tooth within its socket, may pump oral bacteria from gingival crevice into the dental pulp via apical foramen or lateral canals.[39] Even an ideal method of sterilizing the surface of extracted intact teeth would not be capable of preventing this contamination risk.[39] Therefore, dental pulp specimens collected from freshly extracted intact teeth may be contaminated from oral bacteria during extraction process, particularly teeth associated with periodontal infections.

From a homeless patient diagnosed positive for *B. quintana* by blood culture and serum antibody test from 6 previous months, three blood cultures were now negative for *B. quintana* and a maxillary left canine was extracted due to periodontitis. *B. quintana* DNA was PCR-amplified in the dental pulp of this past bacteremia homeless patient whereas negative controls including dental pulp of another patient not exposed to lice remained negative for this microorganism.[40] Also, intact teeth extracted from a patient suffering *S. aureus* bacteremia, yielded *S. aureus* DNA identified from the pulp by PCR depiste a negative culture in the presence of antibiotics (Aboudharam G., unpublished data). These findings suggest

that blood-borne microorganisms can reach the dental pulp which acts as a repository tissue for bacteremic microorganisms.

4.2.2 Dental pulp specimens collected from non-extracted teeth

Whereas the dental pulp flora of endodontic infections is well described,[3] few studies reported the dental pulp microbiota in the intact chamber (Table 1). These studies detected microorganisms from necrotic pulp of the intact teeth. Necrotic pulp was verified by the negativity of the vitality test and radiographic evaluation showing chronic periapical lesions. No patient was under antibiotic or antifungal therapy at the time of sampling. All studies confirmed the intact pulp chamber with crown integrity including absences of carious lesions, fractures, cracks and previous endodontic treatments, as well as without direct oral pulpal communication through fistula, drain, sinus tracts and periodontal pockets. All studies avoided the oral contamination by tooth isolation with rubber dam and chemical desinfection of tooth surfaces before sampling (Table 1). Four studies included sterility controls before opening pulp cavity by bacteria-negative culture of samples taken from the working field after surface desinfection.[41-44] Three studies used culture and biochemical tests as the identification method[41,42,45] whereas culture followed by PCR-sequencing was done in two studies[43,44] and only one study used culture-independent PCR-sequencing method.[46] Three papers[44-46] showed the presence of bacteria in all analysed teeth, two papers[41,42] detected bacteria in 90% - 92% studied teeth and two papers[41,43] isolated yeasts from 4% - 23% teeth (Table 1).

Flora currently identified from intact pulp chamber of non-extracted teeth consists of 44 bacterial genera classified into seven phyla including *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes* and *Deferribacteres* along with fungi. *Firmicutes* (19 genera), *Actinobacteria* (9 genera) and *Proteobacteria* (8 genera) comprise of 82% of genera. Gram-staining and mobility characteristics showed 20 Gram-

negative and 24 Gram-positive bacterial genera along with 22 possibly-motile and 22 non-motile genera (Supplementary table 2). The most abundant genera are *Streptococcus* with 11 species and *Prevotella* with 9 species. Culture identified 36/44 (82%) genera from intact pulp chamber. Only one study[46] using PCR-based molecular method detected 18/44 (41%) genera from intact pulp chamber.

Gram-negative and motile bacteria may play a crucial role in inflammation and necrosis of the dental pulp. Gram-negative bacteria can invade the exposed dentinal tubuli, release endotoxins, toxic agents or antigens from the outer membrane and hence cause inflammatory reactions of the pulp.[47] After the local damage of the pulpal tissue followed by degradation of its defenses, motile bacteria penetrate rapidly into the pulp, proliferate and lead to total pulp necrosis and periapical lesions.[47] This scenario remains hypothetically, as half of bacterial genera detected in necrotic pulp of intact teeth were non-motile or Gram-positive. Therefore, the means of which these latter bacteria colonize the intact pulp chamber need to be further investigated.

Human oral microbiota established by bacterial 16S rRNA gene pyrosequencing[48-51] did not detect some genera isolated from the intact pulp chamber, including *Wolinella* [45], *Stomatococcus*,[42] *Flexistipes*,[46] *Dietzia*, *Rhodococcus*, *Flavobacterium* and *Bacillus*.[44] However, further reviewing the literature showed that periodontal pockets could harbour *Wolinella curva* and *Wolinella recta*,[52] *Stomatococcus mucilaginosus* was an acid-producing oral microorganism with cariogenic potential,[53] *Rhodococcus corynebacteroides* with methylotrophic potential was detected in oral flora[54] and *Flavobacterium indologenes* was isolated from saliva.[55] Moreover, *Bacillus* genus causing serious systemic disease was cultured from periapical periodontitis, suggesting its pathogenic role in endodontic infections.[56] Furthermore, *Flexistipes* species, uncharacterized and

uncultivated spirochetes are now recognized as novel bacteria in endodontic infections and may be potential pathogens involved with etiology of periradicular diseases.[57] Interestingly, *Dietzia* species were only detected in acute infections including septic shock, prosthetic hip infection, aortitis and Gougerot–Carteaud syndrome[58] whereas no study recently reports *Dietzia* genus present in oral cavity. Therefore, this genus detected in the intact pulp chamber may originate from blood-borne infection.

4.3 Archaea

Methanogenic archaea including *Methanobrevibacter smithii,*[59] *Methanobrevibacter oralis,*[60-62] *Methanosarcina mazeii,*[63] *Methanobacterium curvum/congolense*[64] and *Thermoplasmatales*[65] were detected in the subgingival plaque as components of human oral microbiota. Real-time quantitative PCR combined with sequencing and phylogenetic analysis have also identified *M. oralis*-like phylotype in infected root canals.[66] These results have been afterwards confirmed by the observation of archaeal-bacterial communities in both symptomatic and asymptomatic endodontic infections.[67] A further study indicated that archaea were detected in 38.1% of canals with necrotic pulp and were always found in combination with anaerobic bacteria.[68] However, due to lack of the appropriate method for archaeal detection, no study reported the detection of methanogenic archaea in the intact pulp chamber.

4.4 Fungi

The prevalence of yeasts in untreated root canals was found to be 5.7%,[69] but most of these root canals communicated with the oral cavity via coronal restauration leakage, sinus tract or oro-antral fistula. In fact, fungi are considered opportunistic pathogens that cause secondary infections after changing local environment. Discovery of yeasts in necrotic dental pulp led to propose that dental pulp losing its defense capacities after necrosis promotes the growth of fungi.[70] Surprisingly, a study using culture followed

by biochemical tests detected *Candida* sp. in 4% of necrotic pulp specimens collected from intact teeth[41] and another using culture followed by PCR-sequencing identified *C. albicans* in 23% of the intact pulp chamber[43] (Table 1). The latter study also suggested that yeasts may invade the pulpal environment actively or be carried to the region passively via lateral root canals, resorption lacunae, periodontal sulci or probably through dentinal tubles.[43] However, because yeasts are non-motile, the means by which *Candida* sp. may invade the intact pulp chamber is not yet understood.

4.5 Viruses

Human immunodeficiency virus (HIV) PCR-detected in the dental pulp collected from a maxillary right central incisor associated with periodontitis of a HIV-seropositive patient was first reported in 1989.[71] Afterwards, twelve dental pulps were collected from teeth without pulpal disease from eleven HIV-seropositive patients. PCR-based detection of HIV-RNA in 11/12 dental pulp specimens suggested that dental pulp fibroblasts were a likely reservoir for the HIV.[72] However, this study did not state the tooth integrity as well as these teeth non-associated with periodontal pockets. Noteworthy, HIV present in both saliva and gingival crevicular fluid is now confirmed.[73,74] Herpes simplex virus (HSV) was detected by nested-PCR in endodontic infections[75,76] whereas standard PCR did not reveal the presence of HSV-DNA in the dental pulp of HSV-seropositive individuals.[77,78] Indeed, nested-PCR detected HSV in 15/50 necrotic pulp of teeth with carious lesions[76] and this method also detected human herpesviruses in irreversible pulpitis and positive control specimens.[75] Standard PCR instead did not detected human herpesviruses in the dental pulp including healthy pulp collected from intact teeth extracted for orthodontic aims.[77,78] Because of the high risk of contamination associated with nested-PCR, detection of human herpesvirures in the dental pulp should be regarded as questionnable.

Therefore, no study firmly demonstrates viruses present in the intact pulp chamber.

4.6 Non-conventional transmissible agents

Cellular prion protein (PrPc) is present in various tissues including the central nervous system and lymphoreticular tissues.[79] Occasionnally, this protein is transformed into the PrPc isoform (PrPres) characterized by a predominantly beta-structure, insolubility and proteinase resistance, becoming a infectious molecule. PrPres is detectable in human transmissible spongiform encephalopathies including Creutzfeldt-Jakob disease (CJD) and bovine spongiform encephalopathy or scrapie in animals.[79] An experimental study suggested that PrPres of CJD could spread from the dental pulp to the central nervous system and also in the inverse route.[15] However, PrPres was not detected by Western-blotting and the monoclonal 3F4-antibody in the dental pulp collected in 8 CJD patients, despite positive detection in brain tissues.[80] These results were afterwards confirmed: Western blot and immunocytochemistry failed to detect disease-associated PrPres in the dental pulp obtained from non-restored molars of the lower mandibule from two CJD patients.[81] Although PrPres was detectable in trigeminal ganglia and tonsils of all CJD cases, the majority of oral tissues including dental pulp were negative.[82] Because the dental pulp has an extremely rich innervation and a lymphatic system,[83,84] the absence of these non-conventional transmissible agents in the pulp needs also to be further elucidated.

5. Summary and conclusions

In both modern and ancient teeth, a total of 49 bacterial genera along with *Candida* sp. yeasts and *Anelloviridae* viruses were identified in the dental pulp the the intact chamber. Ten bacterial genera comprising *Bacteroides*, *Campylobacter*, *Dialister*, *Eubacterium*, *Lactobacillus*, *Mogibacterium*,

Peptostreptococcus, *Pseudoramibacter*, *Selenomonas* and *Veillonnella* were detected by both culture and PCR from the intact pulp chamber (Figure 3). Molecular detection of the causative agents of some past infections, including *Y. pestis, R. prowazekii, B. quintana, M. tuberculosis, M. leprae* and *S. enterica*, demonstrated blood-borne infection of the dental pulp which may be used as small blood samples for bacteremia diagnosis. However, mechanisms of blood-borne infection of the dental pulp as well as local reactions of the dental pulp during bacteremia need to be further elucidated. Overall, most of microorganisms found in the intact pulp chamber are components of the oral flora, excluding blood-borne infectious agents identified in the ancient teeth and some genus consisting of *Bacillus*, *Flexistipes* and *Dietzia*. These findings suggest a microbial population initially colonizing the dental pulp as bacteria potentially responsible for primary pulpal infections. Recently, high-throughput pyrosequencing showed advantages for discovery of all microorganisms in oral flora[48-51] and endodontic infections.[85] This novel technology could be used to further investigate the intact dental pulp microbiota including archaea and virus, in both modern and ancient teeth.

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Conflict of interest

The authors declare that they have no competing interests.

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Figure 1. Infection sources and contamination pathways of the dental pulp chamber:

1 From carious lesions to pulp chamber directly or via dentine tubules

2 From periodontal pockets to pulp chamber via the apical foramen or lateral canals

3 From bloodstream to pulp chamber via the apical foramen

4 From dental pulp to bloodstream, periapical lesions or fistula via the apical foramen

A: Enamel B: Dentine C: Dental pulp

D: Gum E: Cementum F: Periodontal ligaments

G: Alveolar bone H: Dental neurovascular bundle

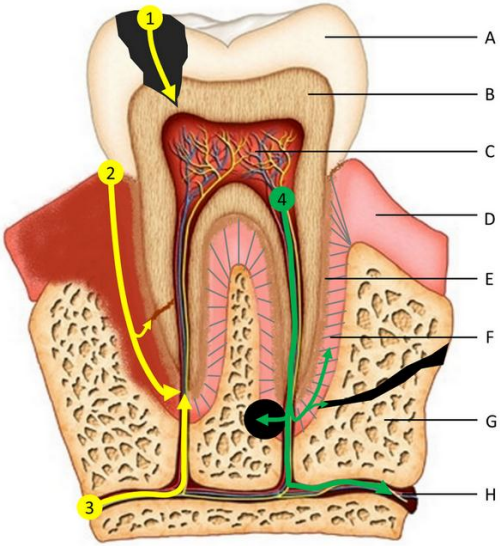

Figure 2: Molecular methods and immunodetection identifying microorganisms from ancient dental pulp specimens collected from European and Mediterranean archeological sites.

△ Immunodetection

□ Molecular detection

Anelloviridae

Bartonella quintana

Mycobacterium tuberculosis

Mycobacterium leprae

Rickettsia prowazekii

Salmonella enterica serovar Typhi

Yersinia pestis

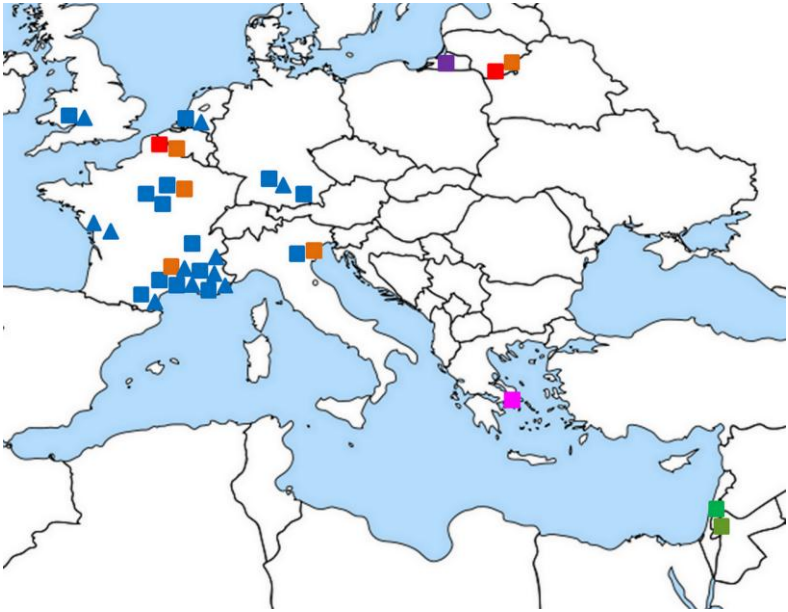

Figure 3: Microorganisms identified from dental pulp of intact teeth and ancient teeth by culture and PCR methods.

Microorganisms detected from intact teeth.

Microorganisms detected from ancient teeth.

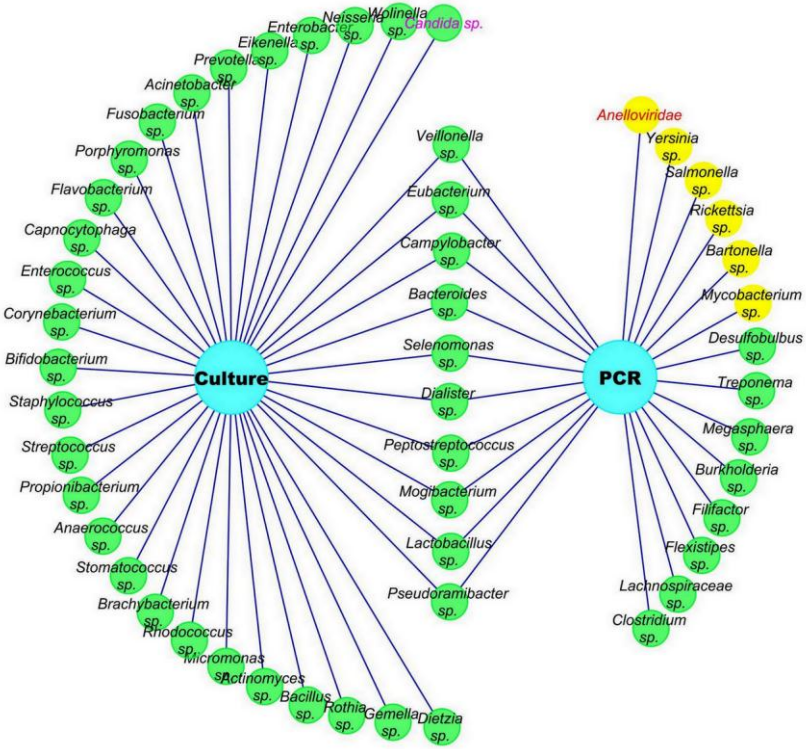

Table 1: Studies detecting microorganisms from dental pulp of intact teeth

References	Pulp vitality	No antibiotics	Intact pulp chamber verification	Prevention of oral contamination	Methods	Results
Sundqvist[45]	Necrotic	Yes	Intact pulp chamber walls	Rubber dam isolation, tooth desinfection	Culture	40 species isolated from 65/65 teeth, the most frequent species: *Fusobacterium nucleatum*
Le Goff et al.[42]	Necrotic	Yes	Crown integrity, no fistula/drain, non-traumatic etiology	Rubber dam isolation, tooth desinfection, sterility controls	Culture	21 species isolated from 18/20 teeth, the most frequent species: *Bacteroides gracilis*
Ferrari et al.[41]	Necrotic	3 months	Without oral communication through fistula or otherwise	Rubber dam isolation, tooth desinfection, sterility controls	Culture	Bacteria and yeasts isolated from 23/25 teeth
Saito et al.[46]	Necrotic	2 months	Absence of carious lesions, fractures, sinus tracts, probing depth > 3 mm	Rubber dam isolation, tooth desinfection	PCR-sequencing	46 taxa identified from 7/7 teeth, the most frequent phyla: *Proteobacteria*
Siqueira et al.[44]	Necrotic	3 months	Absence of carious lesions, fractures, probing depth > 4 mm, endodontic treatment	Rubber dam isolation, tooth desinfection, sterility controls	Culture → PCR-sequencing	52 taxa identified from 32/32 teeth, the most frequent species: *Fusobacterium nucleatum*
Miranda et al.[43]	Necrotic	6 months	No crack, no direct oral pulpal communiacation	Rubber dam isolation, tooth desinfection, sterility controls	Culture → PCR-sequencing	Yeasts identified from 38/168 teeth, the most frequent species: *Candida albicans*

Supplementary table 1: Paleomicrobiological studies using the ancient dental pulp				
Studies	Diseases	Site – date	Methods	Results
Drancourt *et al.* 1998	Plague	Lambesc, Marseille (1590, 1722) – France	Standard PCR – sequencing of rpoB (133-bp) and pla (300-bp) genes	*Y. pestis* in 6/12 teeth
Raoult *et al.* 2000	Plague	Montpellier (14^{th} century) - France	"Suicide PCR" – sequencing of pla (148-bp) gene	*Y. pestis* in 20/23 teeth
Drancourt *et al.* 2004	Plague	Sens, Dreux, Montpellier (5^{th} – 14^{th} century) – France	"Suicide PCR" – sequencing Multiple Spacer Typing (MST) (≤ 300-bp)	*Y. pestis* Orientalis strain in X/19 teeth of 7/8 individuals
Drancourt *et al.* 2005	Trench fever	Roaix, Peyraoutes (2230 – 1950 BC) – France	Nested PCR – sequencing of hbp-E (283-bp) and groEL (269-bp) genes	*B. quintana* in 1/12 teeth
Wiechmann & Grupe 2005	Plague	Aschheim (6^{th} century) – Germany	"suicide PCR" – sequencing of pla (148-bp) gene	*Y. pestis* in 2/6 teeth
Raoult *et al.* 2006	Typhus, trench fever	Vilnius (1812) – Lithuania	Suicide nested-PCR – sequencing of dnaA (141-bp), dnaE (77-bp), hbpE (282-bp) and htrA (113-bp) genes	*R. prowazekii* in 4/72 teeth, *B. quintana* in 7/10 teeth
Papagrigorakis *et al.* 2006	Typhoid fever	Athens (430 – 426 BC) – Greek	"suicide PCR" – sequencing of osmC-clyA (322-bp) and narC (360-bp) genes	*S. enterica* serovar Typhi in 3/3 teeth
Drancourt *et al.* 2007	Plague	Vienne, Martigues, Marseille (7^{th} – 18^{th} century) – France	Suicide nested-PCR – sequencing of glpD (191-bp) gene	*Y. pestis* Orientalis strain in 5/36 teeth
Bianucci *et al.* 2008	Plague	France (1590 – 1722)	Immunodetection of F1 antigen	*Y. pestis* in X/91 teeth of 6/28 individuals
Bianucci *et al.*	Plague	France (16^{th} – 18^{th} century)	Immunodetection of F1 antigen	*Y. pestis* in X/14 teeth of 4/4

2009				individuals
Matheson *et al.* 2009	Tuberculosis, leprosy	Jerusalem (1st century) - Israel	Standard PCR – sequencing of IS6110, RpS12, RLEP, 18kDa-A genes	*M. tuberculosis* in 3/11 teeth, *M. leprae* in 1/11 teeth
Haensch *et al.* 2010	Plague	Netherlands, Germany, England, France (14th -17th century)	Standard PCR – sequencing of pla, caf1, rpoB, glpD, napA, 1.ORI, Branch genes, Immunodetection of F1 antigen	*Y. pestis* in 15/91 teeth
Nguyen-Hieu *et al.* 2010	Typhus, trench fever	Douai (1710 – 1712) - France	Real-time PCR + real-time nested-PCR + "suicide PCR" – sequencing of ITS (102-bp), ipp (152-bp), gap(130-bp) genes and rpmE-$tRNA^{fMet}$ intergenic spacer (115-bp)	*B. quintana* in 1/40 teeth and *R. prowazekii* Madrid E strain in 6/55 teeth
Bedarida *et al.* 2010	DNA-virus	Kaliningrad (1812) - Russia	Standard PCR – sequencing of non-coding region of viral genome	*Anelloviridae* in 1/42 teeth
Tran *et al.* 2011	Plague, trench fever	Venezia (14th -16th century) - Italy	Real-time PCR + suicide nested-PCR – sequencing of ITS (102-bp), pla (98-bp), glpD (191-bp) genes	*B. quintana* in 5/93 teeth and *Y. pestis* in 3/85 teeth
Tran *et al.* 2011	Plague, trench fever	Bondy (11th -15th century) - France	Real-time PCR + suicide nested-PCR - sequencing of ITS (102-bp), pla (98-bp), glpD (191-bp) genes	*B. quintana* in 3/14 teeth and *Y. pestis* in 4/14 teeth
Schuenemann *et al.* 2011	Plague	London (1348 – 1350) - England	Standard and multiplex PCR – Sequencing of pla, caf1M (50 – 60-bp), Branch genes, DNA enrichment – Pyrosequencing	*Y. pestis* in 17/46 teeth Reconstruction of pPCP1

Supplementary table 2 : Microorganisms detected in the intact dental pulp chamber				
Microorganisms in the intact pulp chamber	Gram	Motile	Culture	PCR
Bacteria				
Actinobacteria				
Actinomyces sp./ *A. israelii, A. meyeri, A.naeslundii, A. odontolyticus, A. urogenitalis, A. viscosus*	Positive	Yes/No	+	
Bifidobacterium sp./ *B. dentium*	Positive	No	+	
Brachybacterium sp./ *B. nesterenkovii*	Positive	No	+	
Corynebacterium sp.	Positive	Yes/No	+	
Dietzia sp.	Positive	No	+	
Propionibacterium sp./ *P. acnes, P. granulosum, P. propionicus*	Positive	No	+	
Rhodococcus sp./ *R. rhodochrous*	Positive	No	+	
Rothia sp./ *R. dentocariosa, R. mucilaginosa*	Positive	No	+	
Stomatococcus sp.	Positive	No	+	
Bacteroidetes				
Bacteroides sp./ *B. caccae, B. gracilis, B. ureolyticus*	Negative	Yes/No	+	+
Capnocytophaga sp./ *C. ochracea, C. sputigena*	Negative	Yes	+	
Flavobacterium sp.	Negative	Yes/No	+	
Porphyromonas sp./ *P. endodontalis, P. gingivalis*	Negative	No	+	
Prevotella sp./ *P. buccae, P. buccalis, P. denticola, P. loescheii, P. intermedia, P. marshii, P. melaninogenica, P. oralis, P. salivae*	Negative	No	+	
Firmicutes				
Anaerococcus sp./ *A. viridans, A. prevotii*	Positive	No	+	
Bacillus sp.	Positive	Yes	+	
Clostridium sp.	Positive	Yes/No		+
Dialister sp./ *D. invisus, D. pneumosintes*	Negative	No	+	+
Enterococcus sp./ *E. faecalis*	Positive	No	+	
Eubacterium sp./ *E. alactolyticum, E. brachy, E. lentum, E. nodatum, E. tardum, E. timidum, E. yurii*	Positive	Yes/No	+	+
Filifactor sp./ *F. alocis*	Negative	Yes/No		+
Gemella sp./ *G. haemolysans*	Positive	Yes/No	+	
Lachnospiraceae sp.	Positive	Yes		+
Lactobacillus sp./ *L. catenaforme, L. minutus, L. panis*	Positive	Yes/No	+	+
Megasphaera sp.	Negative	No		+

Micromonas sp./ *M. micros*	Positive	Yes	+	
Mogibacterium sp./ *M. neglectum*	Positive	No	+	+
Peptostreptococcus sp./ *P. anaerobius, P. niger, P. prevotii, P. micros*	Positive	No	+	+
Pseudoramibacter sp./ *P. alactolyticus*	Positive	No	+	+
Selenomonas sp./ *S. sputigena*	Positive	Yes	+	+
Staphylococcus sp./ *S. aureus, S. epidermidis, S. pasteuri, S. saccharolyticus*	Positive	No	+	
Streptococcus sp./ *S.anginosus , S. constellatus, S. gordonii, S. infantis, S. intermedius, S. mitis, S. morbillorum, S.mutans, S. oralis, S. parasanguinis, S. sanguinis*	Positive	Yes/No	+	
Veillonella sp./ *V. parvula*	Negative	Non	+	+
Fusobacteria				
Fusobacterium sp./ *F. nucleatum, F. periodonticum*	Negative	Yes/No	+	
Proteobacteria				
Acinetobacter sp.	Negative	No	+	
Burkholderia sp./ *B. fungorum, B. phenazinium*	Negative	Yes		+
Campylobacter sp./ *C. curvus, C. gracilis, C. hongkongensis, C. rectus*	Negative	Yes	+	+
Desulfobulbus sp.	Negative	Yes/No		+
Eikenella sp./ *E.corrodens*	Negative	No	+	
Enterobacter sp./ E. agglomerans	Negative	Yes	+	
Neisseria sp./ *N. sicca*	Negative	No	+	
Wolinella sp./ W.curva, W. recta	Negative	Yes	+	
Spirochaetes				
Treponema sp./ *T. socranskii*	Negative	Yes		+
Deferribacteres				
Flexistipes sp.	Negative	No		+
Fungi				
Candida sp./ *C. albicans, C. glabrata, C. magnoliae, C. parapsilosis*			+	

Chapitre III

Heat degradation of eukaryotic and bacterial DNA: an experimental model for paleomicrobiology

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Chapitre III – Avant propos

Quelques auteurs ont suggéré que l'ADN bactérien est plus résistant que l'ADN humain dans les échantillons anciens. Dans cette étude, nous avons utilisé la chaleur, agent dégradant de l'ADN le plus souvent observé dans l'environnement pour créer un modèle expérimental, imitatif et accéléré de la dégradation naturelle de l'ADN dans les échantillons anciens, et pour comparer la dégradation de l'ADN bactérien et eucaryote. Les macrophages murins J774, *Mycobacterium smegmatis* et les macrophages infectés par *M. smegmatis* ont été soumis à la chaleur sèche à 90°C suivant une cinétique d'une à 48 heures. L'amplification de l'ADN par PCR en temps réel a été réalisée avec les systèmes d'amorces Rpb2 et RpoB combinés aux sondes TaqMan. Les systèmes d'amorces sélectionnés ont pu amplifier les fragments de l'ADN de différentes tailles de 150-paires de bases (-pb) jusqu'à 750-pb. Le système d'amorce Rpb2 a amplifié spécifiquement les fragments de différentes tailles de l'ADN des macrophages J774 et le système d'amorce RpoB a amplifié également spécifiquement les fragments de l'ADN de *M. smegmatis.* Toutes les expériences ont été répétées 3 fois et les résultats négatifs ont été confirmés par 3 fois. Les résultats ont montré que les longs fragments de 750-pb de l'ADN des mycobactéries étaient résistants à la chaleur sèche jusqu'à 48h d'incubation à 90°C tandis que l'ADN des macrophages J774 a été dégradé en petits fragments de 450-bp pendant la même durée d'incubation. Dans le modèle des macrophages infectés par les mycobactéries, une dégradation de l'ADN des macrophages J774 en fragments de 450-bp a été également observée par contre on a observé une préservation des fragments de 750-pb de l'ADN de *M. smegmatis* après 48h d'incubation à 90°C. Ces différences sont statistiquement significatives (test ANOVA, $p < 0.05$). Pour l'application dans la paléomicrobiologie, ces résultats suggèrent que les méthodes

moléculaires basées sur les techniques de PCR peuvent détecter les fragments plus longs de l'ADN bactérien à partir des échantillons anciens.

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SHORT REPORT Open Access

Heat degradation of eukaryotic and bacterial DNA: an experimental model for paleomicrobiology

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Abstract

Background: Theoretical models suggest that DNA degradation would sharply limit the PCR-based detection of both eukaryotic and prokaryotic DNA within ancient specimens. However, the relative extent of decay of eukaryote and prokaryote DNA over time is a matter of debate. In this study, the murine macrophage cell line J774, alone or infected with *Mycobacterium smegmatis* bacteria, were killed after exposure to 90°C dry heat for intervals ranging from 1 to 48 h in order to compare eukaryotic cells, extracellular bacteria and intracellular bacteria. The sizes of the resulting mycobacterial *rpo*B and murine *rpb*2 homologous gene fragments were then determined by real-time PCR and fluorescent probing.

Findings: The cycle threshold (Ct) values of PCR-amplified DNA fragments from J774 cells and the *M. smegmatis* negative controls (without heat exposure) varied from 26–33 for the J774 *rpb*2 gene fragments and from 24–29 for *M. smegmatis rpo*B fragments. After 90°C dry heat incubation for up to 48 h, the Ct values of test samples increased relative to those of the controls for each amplicon size. For each dry heat exposure time, the Ct values of the 146-149-bp fragments were lower than those of 746-747-bp fragments. During the 4- to 24-h dry heat incubation, the non-infected J774 cell DNA was degraded into 597-bp *rpb*2 fragments. After 48 h, however, only 450-bp *rpb*2 fragments of both non-infected and infected J774 cells could be amplified. In contrast, the 746-bp *rpo*B fragments of *M. smegmatis* DNA could be amplified after the 48-h dry heat exposure in all experiments. Infected and non-infected J774 cell DNA was degraded more rapidly than *M. smegmatis* DNA after dry heat exposure (ANOVA test, $p < 0.05$).

Conclusion: In this study, mycobacterial DNA was more resistant to dry-heat stress than eukaryotic DNA. Therefore, the detection of large, experimental, ancient mycobacterial DNA fragments is a suitable approach for paleomicrobiological studies.

Keywords: Ancient DNA, DNA degradation, Bacterial DNA, Eukaryotic DNA, *Mycobacterium*, Real-time PCR

Findings

Introduction

The seminal demonstration that nuclear DNA could be cloned from a 2,400-year-old Egyptian mummy [1] founded molecular paleontology and paleomicrobiology [2,3]. However, the cumulative experience over the past few decades indicates that the detection of ancient DNA (aDNA) could be limited by DNA degradation influenced by pH and humidity of the burial site. Heat, ultraviolet rays and oxidative agents are also proposed to contribute to the alteration of the chemical nature of nucleic acid bases and the degradation of DNA in buried human and animal remains [4-8]. Based on theoretical models of DNA degradation [4,9,10], some authors have questioned the long-term stability of DNA and suspected that some paleomicrobiological detections may have resulted from the contamination of samples with modern DNA [5,11]. Studies have demonstrated aDNA to be degraded into < 150-bp nuclear and < 400-bp mitochondrial fragments [12,13]. Experimental models of DNA degradation have used purified DNA [14-16]

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because few studies have assessed the degradation of DNA within cells [17]. Moreover, these early studies used eukaryotic DNA. Indeed, the potential degradation of prokaryotic aDNA was only extrapolated from the data obtained from eukaryotic aDNA without experimental validation. Some authors have proposed, however, that in ancient, buried specimens, bacterial DNA may be more resistant to decay over time than human DNA [18,19]. In this study, we evaluated experimental DNA decay in both eukaryotic and prokaryotic cells.

Dry heat degradation of murine macrophage cell line J774 DNA and M. smegmatis DNA

One-hour incubation of *M. smegmatis* and J774 cells at 90°C resulted in cell death as indicated by the absence of subculture. All blank control PCR reactions were negative. The cycle threshold (Ct) values of PCR-amplified fragments from J774 DNA and *M. smegmatis* negative control DNA (without heat exposure) varied from 26–33 for J774 cell *rpb*2 fragments and 24–29 for *M. smegmatis rpo*B fragments. For test samples exposed to 90°C dry heat for intervals ranging from 1 to 48 h, the Ct values of PCR-amplified fragments were observed to increase relative to those of controls at each amplicon size tested. Furthermore, for each dry heat exposure time, the Ct values of the shorter 146-149-bp fragments were lower than those of the longer 746-747-bp fragments (Additional file 1: Table S1). During the 4- to 24-h dry heat exposures, J774 cell DNA degraded into 597-bp fragments. After 48 h of exposure, however, only 450-bp *rpb*2 fragments of J774 cell DNA could be amplified. In contrast, 746-bp *rpo*B fragments of *M. smegmatis* DNA could be amplified even after 48 h of dry heat exposure (Figure 1). The DNA degradation of J774 cells was more rapid than that of *M. smegmatis* through 1 – 48 – hour exposure to dry heat (ANOVA test, $p < 10^{-3}$, Additional file 2: Table S2).

Dry heat degradation of M. smegmatis-infected J774 cell DNA

In the experiments utilizing intracellular bacteria, all blank controls were negative. *M. smegmatis*-infected J774 cells not exposed to dry heat served as negative controls, and the Ct values of PCR-amplified DNA varied from 23 – 30 for J774 fragments and 27 – 28 for *M. smegmatis* fragments. After exposure to 90°C dry heat for durations ranging from 1 to 48 h, the Ct values of test samples were observed to increase relative to those of controls for each amplicon size. Additionally, for each dry heat exposure time, the Ct values of the shorter 146-149-bp fragments were lower than those of the longer 746-747-bp fragments (Additional file 3: Table S3). After 48 h of dry heat exposure, only the 450-bp fragments of infected J774 cell DNA could be amplified. In contrast, the 746-bp fragments of intracellular *M. smegmatis* DNA could be amplified following the same 48-h dry heat exposure (Figure 2). The DNA degradation of the infected-J774 cells was more rapid than that of intracellular *M. smegmatis* through 2 – 12 – hour and 48 – hour exposure to dry heat (ANOVA test, $p < 0.05$, Additional file 2: Table S2). There was no statistically significant difference in the degradation of J774 cell DNA, between *M. smegmatis*-infected and non-infected cells (ANOVA test, $p > 0.05$, Additional file 2: Table S3).

The data presented in this manuscript can be interpreted as both authentic and biologically relevant. All blank controls used in our PCR-based experiments were negative. Additionally, the control cells not exposed to dry heat yielded the expected results. Macrophages and mycobacteria were exposed to dry heat in parallel, and the *rpo*B gene of mycobacteria was assessed in parallel with its homolog, the *rpb*2 gene of murine macrophages. Reproducible results were observed across experiments performed in triplicate.

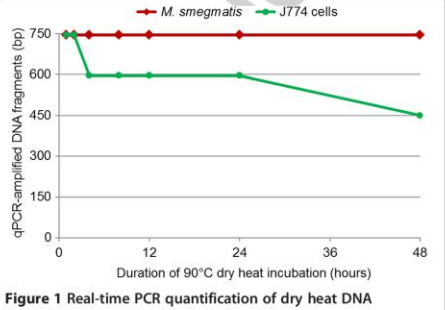

Figure 1 Real-time PCR quantification of dry heat DNA degradation of *M. smegmatis* and J774 cells.

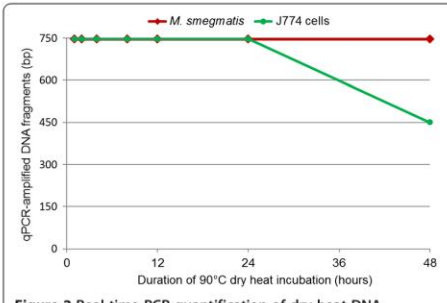

Figure 2 Real-time PCR quantification of dry heat DNA degradation of *M. smegmatis*-infected J774 cells.

The dry heat used in this study has been used previously to assess experimental degradation of purified DNA [16,17] and is the most common environmental agent that may cause DNA damage in dead cells. Thus, our experiments utilizing dry heat exposure mimic an accelerated natural DNA degradation process. Furthermore, in this study, dry heat was applied to cellular DNA rather than purified DNA. We verified that these conditions killed mycobacteria. However, the experimental design of the study did not allow us to rule out the hypothesis that viable non-culturable mycobacteria persisted into infected macrophages [20], possibly interfering with macrophage DNA degradation. Additionally, all previously published experiments have used agarose gel electrophoresis to monitor DNA degradation [14-17]. This method of evaluation is imprecise, as it relies on the visual observation of smears and only provides estimates as to the extent of DNA degradation. In our study, we used real-time PCR in combination with TaqMan® fluorescent probes to accurately monitor the size of PCR-amplifiable fragments of mycobacterial and eukaryotic DNA following exposure to heat stress. *M. smegmatis* was chosen because it has a lipid-rich cell wall similar to that of *Mycobacterium tuberculosis*, a pathogen previously investigated in paleomicrobiological studies [3,18]. Furthermore, *M. smegmatis*-infected J774 cells were used as an experimental model with which to assess DNA degradation of obligate intracellular bacteria. This model of particular interest because *Rickettsia prowazekii* and *Mycobacterium leprae*, which are obligate intracellular bacteria, have been detected in ancient specimens [21-24]. Our studies assessing experimental and comparative degradation of cellular DNA in both bacteria and eukaryotic cells are the first in the literature.

Several criteria have been published to authenticate aDNA-based data [2,11]. These include the absence of a positive control, negativity of negative controls, sequencing all PCR amplicons, amplification and sequencing of a second target, originality of the ancient sequences and reproducibility of results in at least two independent laboratories. The data presented here are consistent with previously published paleomicrobiological observations. DNA fragments exceeding 350-bp have been PCR-amplified from several ancient bacterial pathogens, including *M. tuberculosis* and *M. leprae*, from a number of ancient, buried individuals (Figure 3, Additional file 4: Table S4). Bone, mummified tissues (e.g. mummy, skull, lung, pleura, and rib) and dental pulp are often used for these types of paleomicrobiological investigations (Figure 3), although aDNA is more easily extracted from soft tissues than from calcified ones. The data reported herein cannot be extrapolated to other bacteria, nevertheless DNA fragments larger than 250-bp from infectious agents have been successfully PCR-amplified from ancient dental pulp specimens [24-31]. This finding supports the hypothesis that dental pulp is a good source of genetic material for DNA-based paleomicrobiological studies [32]. The published data indicate that the average eukaryotic DNA fragment size in these experiments does not correlate with the age of ancient specimens (Figure 3), suggesting that DNA degradation occurs relatively soon after cell death [4,12]. Indeed, 250-bp average fragments have been sequenced from both a 30,000-year-old permafrost sediment [33] as well as a 40,000-year-old cave bear bone [34]. Interestingly, most large DNA fragments recovered from permafrost sediment are comprised of mostly bacterial DNA, supporting the idea that bacterial DNA was better preserved than eukaryotic DNA in ancient specimens.

Conclusions

Field observations, along with the experimental data presented here, suggest that mycobacterial DNA is more resistant than eukaryotic DNA to taphonomic degradation. Bacterial DNA fragmentation could be offset by DNA repair activities [35,36]. The measurement of carbon dioxide release from bacteria embedded in permafrost for 500,000 years found that bacterial metabolic activity ensured survival and DNA repair capacity [37]. Additionally, thick cell walls, like those of mycobacteria, could protect bacterial DNA from certain degrading agents [18,19]. The dogma that ancient bacterial DNA is fragmented to an extent where only targets shorter than 200-bp can be detected [38] is not supported by either the experimental data or the high-throughput pyrosequencing observations. Large bacterial DNA fragments can be detected from ancient buried specimens without enzymatic reparation [39].

Methods

Culture of murine macrophage cell line J774 and M. smegmatis

The murine macrophage J774 (ATCC TIB 67) cells were cultured in GIBCO® 1X DMEM culture medium (Invitrogen, Carlsbad, USA) supplemented with 10% heat-decomplemented fetal calf serum (Seromed, Strasbourg, France) and 1% glutamine (Seromed) at 37°C with 5% CO_2 for 3 days. *M. smegmatis* mc2 (ATCC 700084) was cultured in trypticase-soy-casein broth (European Pharmacopia IV, Strasbourg, France) supplemented with 0.5% Tween 80 (European Pharmacopia IV) at 37°C for 10 days. For the co-culture experiment, 1.8 mL of a 10^6 mycobacteria/mL suspension were incubated with 15 mL of a 10^5 J774 cells/mL suspension at 37°C under 5% CO_2 atmosphere for 4 h. The infected J774 cell monolayer was then washed two times with 15 mL sterile phosphate buffered saline (PBS) before 15 mL fresh culture medium supplemented with 1% streptomycin

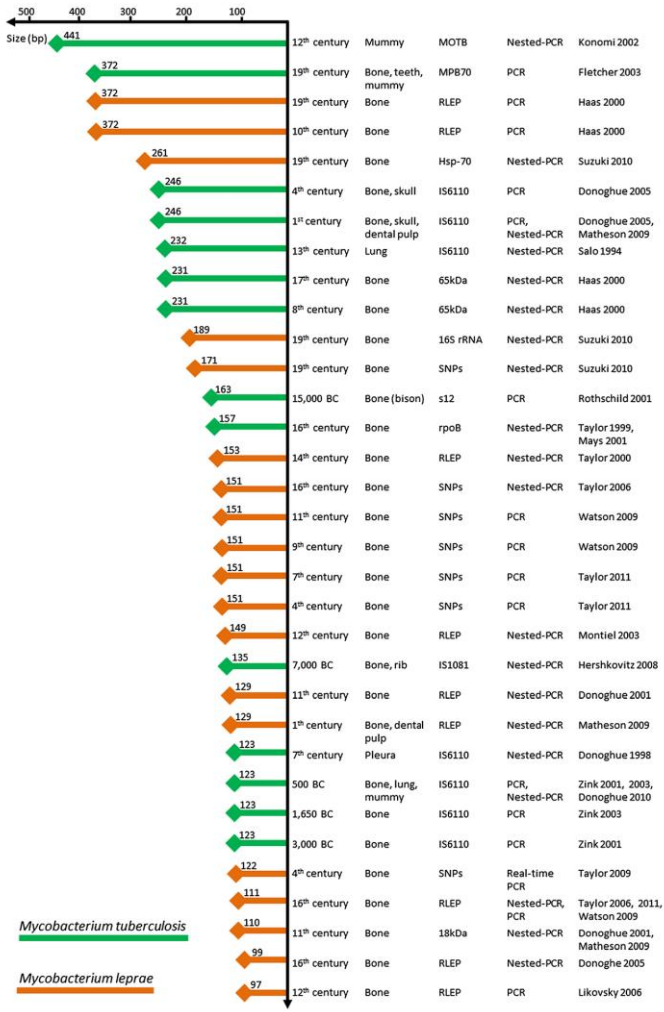

Figure 3 Mycobacterial DNA fragments PCR-amplified from ancient specimens (References included in the Additional file 4: Table S4).

(Panpharma, Fougères, France) was added for 2 h, eliminating any extracellular mycobacteria [40]. The infected-cell monolayer was then washed two times with 15 mL sterile PBS before 15 mL of fresh culture medium was added. The monolayer was incubated at 37°C with 5% CO_2 for 24 h. Infection of the J774 cell layer was monitored by Ziehl-Neelsen staining. The viability of *M. smegmatis* mycobacteria and J774 cells after one-hour incubation at 90°C was assessed by subculture as described above.

Experimental degradation of DNA

200 μL suspensions of 2.10^4 J774 cells/mL, 2.10^5 *M. smegmatis*/mL or 2.10^4 *M. smegmatis*-infected J774 cells/

mL were incubated in parallel at 90°C in a dry heat incubator (Techne Dri-Block®, Staffordshire, UK) for 1, 2, 4, 8, 12, 24 or 48 h. All experiments were conducted in triplicate. The cell suspensions not exposed to dry heat were included as negative controls. The DNA was extracted by adding 0.3 g of 106-μm glass beads (Sigma Aldrich, Steinheim, Germany) to 200 μL J774 cells or to *M. smegmatis* or *M. smegmatis*-infected J774 cells in 1.5 mL Eppendorf tubes. The cell suspensions were homogenized in a FastPrep-24 Instrument (MP Biomedicals Europe) 3 times for 20 s at 4 m/s and were then centrifuged at 16,045 x g for 3 min. DNA extraction was then performed using the QIAamp® DNA Mini kit (Qiagen, Hilden, Germany) following a modified protocol. Briefly, 200 μL ATL buffer and 20 μL proteinase K were added to each cell tube. The cell suspensions were vortexed for 15 s and then incubated at 56°C for 45 min. The 420-μL supernatant was transferred to a new Eppendorf tube and mixed with 200 μL absolute ethanol by vortexing for 15 s. The 620-μL mixture was transferred to a NucleoSpin column, and all following steps were conducted according to the QIAamp® DNA Mini Kit protocol. The resulting DNA was diluted with 60 μL AE buffer.

Real-time PCR measurements

Two PCR primer systems were designed using the software Perlprimer version 1.1.6 [41]. The Rpb2.J774mur primer system specifically amplified sequences of different sizes (e.g., 146-bp, 298-bp, 450-bp, 597-bp and 747-bp) of the J774 *rpb*2 gene (gi|161898209|gb|EF536008.1|), and primer system RpoB.Msmeg amplified sequences of different sizes (149-bp, 298-bp, 444-bp, 599-bp and 746-bp) of the *M. smegmatis rpo*B gene (gi|34595742|gb|AY262735.1|). Two fluorescent probes, Rpb2.J774mur-TaqMan and RpoB.Msmeg-TaqMan, were designed to hybridize to the PCR-amplified *rpb*2 and *rpo*B fragments, respectively (Figure 4, Additional file 5: Table S5). Amplifications were performed in Stratagene™ MX3000P (Agilent Technologies Company, La Jolla, CA, USA) and CFX96™ Real-Time Systems (Bio-Rad, Singapore) using 10 μL reaction mixture (Quantitech, Qiagen), 2 μL sterile water, 2 μL Taqman® probe (Applied Biosystems, Villebon-sur-Yvette, France), 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM) and 5 μL DNA by under the following PCR conditions: 15-min activation at 95°C and 40 cycles of 30-s denaturation at 95°C, 45-s hybridization at 62°C and 90-s elongation at 72°C. Ten negative controls (using DNA extracted from cells without 90°C dry heat exposure) were included in every batch. Additionally, ten blank controls (PCR-mix with sterile water instead of DNA) were also included. For each test sample, real-time PCR amplification was conducted in triplicate. Cut-offs were defined empirically; for each amplicon size, a test sample was considered as PCR-negative if its Ct value was greater than the Ct value of the negative control plus 12 (4,096 times of decreasing DNA concentration) or if its Ct value was nil. The Ct value of the test sample (Ctx) being 41 was delivered for no PCR-amplification or nil Ct values. PCR-detections of degraded DNA were confirmed as negative when all of three replicates were negative.

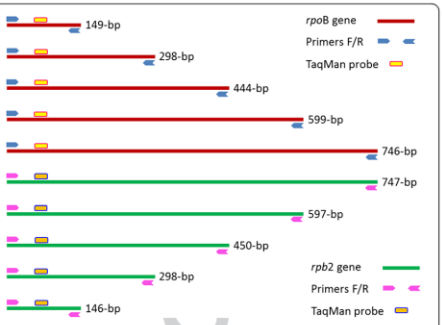

Figure 4 Two primer systems with fluorescent probes specifically amplifying different fragments of the mycobacterial *rpo*B gene and its murine homolog *rpb*2.

Statistical analyses

The value of the negative control (Ct0) was used as baseline. For each amplicon size, the DNA degradation was calibrated by Ctx – Ct0. The ANOVA test was used to compare the means of (Ctx – Ct0) values.

Additional files

Additional file 1: Table S1. Average Ct values of *rpb*2 and *rpo*B amplified-fragments of J774 cells and *M. smegmatis*.

Additional file 2: Table S2. ANOVA tests comparing means of (Ctx – Ct0) values.

Additional file 3: Table S3. Average Ct values of *rpb*2 and *rpo*B amplified-fragments of *M. smegmatis*-infected J774 cells.

Additional file 4: Table S4. References of mycobacterial DNA PCR-amplifications from ancient specimens (Figure 3) [1-24].

Additional file 5: Table S5. Primers and Taqman® probes used for real-time PCR quantification of J774 cell *rpb*2 and *M. smegmatis rpo*B genes.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TNH did the experiments, analyzed the data and drafted the manuscript. GA participated in the overall design and finalized the draft of the manuscript. MD designed the study, analyzed the data and finalized the draft of the manuscript. All the authors read and approved the final version of the manuscript.

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Additional file 1: Table S1: Average Ct values of *rpb*2 and *rpo*B amplified-fragments of J774 cells and *M. smegmatis.*

	J774 cells DNA – *rpb*2 gene					***M. smegmatis* DNA – *rpo*B gene**				
	146-bp	**298-bp**	**450-bp**	**597-bp**	**747-bp**	**149-bp**	**298-bp**	**444-bp**	**599-bp**	**746-bp**
Controls	25.26	26.77	27.66	28.95	32.14	23.98	25.39	26.45	27.22	28.42
1 hour	28.93	30.89	32.33	33.25	37.41	22.59	24.23	23.81	25.23	26.49
2 hours	30.51	33.67	35.67	38.13	39.85	22.37	23.81	24.45	26.36	27.82
4 hours	31.89	35.01	35.99	39.00	-	22.11	23.68	24.14	25.35	26.89
8 hours	33.71	34.51	35.83	37.80	-	22.30	24.26	25.26	26.66	28.17
12 hours	33.42	34.77	35.78	38.60	-	24.24	26.42	26.77	28.35	29.77
24 hours	32.87	35.82	36.01	38.64	-	26.02	28.01	28.35	29.73	31.36
48 hours	33.01	35.83	37.53	-	-	27.80	30.31	30.03	31.90	34.56

Additional file 3: Table S3: Average Ct values of *rpb*2 and *rpo*B amplified-fragments of *M. smegmatis*-infected J774 cells.

	J774 cells DNA – *rpb*2 gene					***M. smegmatis* DNA – *rpo*B gene**				
	146-bp	**298-bp**	**450-bp**	**597-bp**	**747-bp**	**149-bp**	**298-bp**	**444-bp**	**599-bp**	**746-bp**
Controls	22.38	24.06	23.46	24.49	29.18	26.67	26.71	26.67	27.56	27.94
1 hour	24.36	26.01	25.64	26.72	30.09	28.60	28.61	28.79	29.52	29.92
2 hours	27.60	29.63	29.56	30.75	31.14	29.50	29.52	29.79	30.60	30.89
4 hours	0.34	32.48	33.86	34.99	35.54	30.12	30.38	30.58	31.47	31.55
8 hours	30.50	34.79	34.37	36.14	36.49	30.10	30.65	30.89	31.75	32.60
12 hours	30.40	33.71	33.60	34.64	36.06	32.08	32.49	30.09	32.29	32.84
24 hours	27.32	30.16	30.76	32.40	33.24	28.92	29.33	29.59	30.60	31.33
48 hours	30.02	34.56	34.88	-	-	31.43	32.22	31.36	34.96	34.15

Additional file 2: Table S2: ANOVA tests comparing means of (Ctx – Ct0) values.

	J774 cells versus *M. smegmatis* **(p-value)**	Infected J774 cells versus Intracellular *M. smegmatis* **(p-value)**	J774 cells versus Infected J774 cells **(p-value)**	*M. smegmatis* versus Intracellular *M. smegmatis* **(p-value)**
1 hour	4.52 vs -1.33 **(0.000)**	1.98 vs 2.00 **(0.914)**	4.52 vs 1.98 **(0.000)**	-1.33 vs 2.00 **(0.000)**
2 hours	7.50 vs -0.81 **(0.000)**	5.64 vs 2.95 **(0.000)**	7.50 vs 5.64 **(0.001)**	-0.81 vs 2.95 **(0.000)**
4 hours	9.02 vs -1.36 **(0.000)**	9.28 vs 3.67 **(0.000)**	9.02 vs 9.28 **(0.772)**	-1.36 vs 3.67 **(0.000)**
8 hours	9.10 vs 0.51 **(0.000)**	10.25 vs 4.07 **(0.000)**	9.10 vs 10.25 **(0.214)**	0.51 vs 4.07 **(0.000)**
12 hours	9.17 vs 2.29 **(0.000)**	9.34 vs 6.64 **(0.028)**	9.17 vs 9.34 **(0.836)**	2.29 vs 6.64 **(0.000)**
24 hours	9.14 vs 3.81 **(0.000)**	9.02 vs 6.26 **(0.151)**	9.14 vs 9.02 **(0.925)**	3.81 vs 6.26 **(0.109)**
48 hours	10.42 vs 5.89 **(0.000)**	10.75 vs 6.82 **(0.005)**	10.42 vs 10.75 **(0.743)**	5.89 vs 6.82 **(0.416)**

Additional file 5: Table S5: Primers and Taqman® probes used for real-time PCR quantification of J774 cell *rpb*2 and *M. smegmatis rpo*B genes.

Systems primers	***Name***	***Sequence (5' – 3')***	***Amplicon***	***Tm***
	Rpb2.J774mur-F	CTATAACCTGAATGTAGCAAGC		
	Rpb2.J774mur-R1	CCCAATGAGGTGCTAGACTC	146-bp	
	Rpb2.J774mur-R2	AATATTAACCAAGTTTAGAAACGC	298-bp	
Rpb2.J774mur	Rpb2.J774mur-R3	CCTAGCACGAGAATGAATTTTG	450-bp	62°C
	Rpb2.J774mur-R4	CATCACTCGCCGCCTCTAC	597-bp	
	Rpb2.J774mur-R5	TATATCCCAAGTTTTATGAAGGG	747-bp	
	Rpb2.J774mur-TaqMan	6FAM-TGCAAGATGTCATAGGCATACAGC-TAMRA		
	RpoB.Msmeg-F	TCTCCGAGATCATGATGGGC		
	RpoB.Msmeg-R1	CTCCTTGAAGAACAGGTTCTC	149-bp	
	RpoB.Msmeg-R2	GTCTGACCCTCGTGCAGAC	298-bp	
RpoB.Msmeg	RpoB.Msmeg-R3	CACGCACGACACGCTCCAT	444-bp	62°C
	RpoB.Msmeg-R4	ACGCTTGTGGGTCAGACCC	599-bp	
	RpoB.Msmeg-R5	CACCGACAGCGAACCGATC	746-bp	
	RpoB.Msmeg-TaqMan	6 FAM-ACGAGGCCCTGCTCGACATCTA-TAMRA		

Additional file 4: Table S4: References of mycobacterial DNA PCR-amplifications from ancient specimens (Figure 3).

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Chapitre IV

Evidence of a louse-borne outbreak involving typhus in Douai, 1710-1712 during the war of Spanish succession

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Chapitre IV – Avant propos

Les maladies infectieuses ont tué plus de soldats pendant les guerres que les armes. Une approche moléculaire et anthropologique permet à partir de la pulpe dentaire d'identifier les causes probables de ces maladies. Récemment, un charnier, daté du début du 18ème siècle, a été exploré à Douai en France, ville assiégée de 1710 à 1712 pendant la guerre de la succession d'Espagne. L'étude anthropologique des restes humains des individus, en dépit du jeune âge a conclu que leur décès pourrait être compatible avec une maladie transmise par les poux. A partir de ce charnier et de 11 autres, une étude moléculaire a été effectuée. L'analyse moléculaire de l'ADN pulpaire et la détection de *Bacillus anthracis*, *Borrelia recurrentis*, *Bartonella quintana*, *Rickettsia prowazekii*, *Salmonella enteria* serovar Typhi, *Yersinia pestis* et *Poxvirus* a été effectuée par PCR en temps réel sur 1192 dents anciennes de ces 12 charniers. Ensuite, orientée par les données anthropologiques, une recherche plus spécifique de *R. prowazekii* a été réalisée sur des dents provenant de Douai par plusieurs techniques de PCR. La détection de fragments de 206-pb et 152-pb du gène spécifique *Invertase Pin-like protein* a été effectuée par PCR en temps réel emboitée. La confirmation des résultats obtenus a été réalisée par «PCR suicide» emboitée avec la détection de fragments de 187-pb et 130-pb du gène spécifique *Glutamine amidotransferase like protein*. Enfin le génotypage de *R. prowazekii* a été réalisé avec la recherche d'une séquence intergénique rpmE-tRNAfMet par PCR en temps réel. Tous les produits de PCR ont été séquencés et comparés avec les séquences disponibles dans GenBank. Dans le premier test, *B. quintana* a été détectée dans 1/40 dents provenant du charnier de Douai. Puis à partir de 55 dents appartenant à 21 individus retrouvées à Douai, nous avons détecté et confirmé la présence de l'ADN de *R. prowazekii* dans 6/55 pulpes dentaires (11%) collectées de 6/21

squelettes (28.6%). Enfin le génotypage de *R. prowazekii* a donné le type B souche Madrid E. La détection moléculaire de *R. prowazekii* dans la pulpe dentaire a confirmé scientifiquement l'épidémie de typhus suspectée dans le village de Douai au 18ème siècle (1702-1712). C'est la plus ancienne détection de *R. prowazekii* et ces résultats supportent l'hypothèse que le typhus a été introduit en Europe par les soldats espagnols au retour des conquêtes en Amérique.

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Evidence of a Louse-Borne Outbreak Involving Typhus in Douai, 1710-1712 during the War of Spanish Succession

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Abstract

Background: The new field of paleomicrobiology allows past outbreaks to be identified by testing dental pulp of human remains with PCR.

Methods: We identified a mass grave in Douai, France dating from the early XVIIIth century. This city was besieged during the European war of Spanish succession. We tested dental pulp from 1192 teeth (including 40 from Douai) by quantitative PCR (qPCR) for *R. prowazekii* and *B. quintana*. We also used ultra-sensitive suicide PCR to detect *R. prowazekii* and genotyped positive samples.

Results and Discussion: In the Douai remains, we identified one case of *B. quintana* infection (by qPCR) and *R. prowazekii* (by suicide PCR) in 6/21 individuals (29%). The *R. prowazekii* was genotype B, a genotype previously found in a Spanish isolate obtained in the first part of the XXth century.

Conclusion: Louse-borne outbreaks were raging during the XVIIIth century; our results support the hypothesis that typhus was imported into Europe by Spanish soldiers from America.

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Introduction

As mentioned by Zinsser, infectious illness has killed more soldiers during war than weapons [1]. The current genetic tools make it possible in a certain number of cases to identify the probable causes of epidemics [2]. The combination of an anthropological approach that identifies burials from catastrophes with a molecular approach that makes it possible to identify the genes of bacteria in dental pulp has developed recently into the framework of a new speciality called paleomicrobiology [3]. Thanks to these elements, we found that the Justinian plague, like the great plague of the Middle Ages, was due to *Yersinia pestis* Orientalis [4–6]. We also identified the presence of *Bartonella quintana* in very ancient samples [7]. More recently, we showed that some of the soldiers of the Grand Army that died in Vilnius after the passage of the Bérézina river died of diseases transmitted by lice: *Bartonella quintana* and *Rickettsia prowazekii* [8].

Recently, a mass grave dating back to the 18th century was explored in Douai, France. The city of Douai was besieged from 1710 to 1712, being successively occupied by the French and then the Dutch and then retaken by French in 1712 during the war of Spanish succession (Figure 1). These events were in the framework of a generalised European war, with France and Spain opposing the other nations, the battle being carried out on the French side by Louis XIV "Le Grand Monarque." The investigation of this mass grave showed that few skeletons presented lesions compatible with weapon wounds. This led to the assumption that a certain number of these skeletons were caused by an epidemic that occurred during the siege. Indeed, the medical condition of the men was very bad in spite of their young age. This could be consistent with an epidemic of diseases transmitted by lice.

Materials and Methods

Source of the materials

Douai is a village located in northern France. It was disputed between France and the Great Alliance of La Haye between 1702 and 1712 [9,10]. The site of the street Martin-du-Nord was discovered during building construction in 1981 (Figure 2). A total of twelve multiple burials and four individual graves have been uncovered at this site. The graves appeared to be scattered over the plot in various directions (Figure 3), and the individuals were deposited head-to-foot into exiguous pits, a characteristic of disaster graves linked to sudden and massive mortality. This type of burial can also occur during an epidemic [11,12]. The demographic profile of young males as well as historical documents suggested a military installation. Five individuals with evidence of traumatic injuries were buried in a single pit. The cause of death could not be attributed to trauma in the other individuals, and 21 such individuals were studied herein, from

Figure 1. Representation of the bi siege of Douai in an Almanach.
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Figure 2. General view of the burial site of Douai.
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whom a total of 55 teeth were used for the molecular investigations [13,14] (Figure 4).

High-throughput detection of pathogens

Dental pulp recovered as previously described [4] was incubated overnight at 56°C with 600 µL ATL buffer and 50 µL proteinase K. DNA was then extracted by using QIAamp Media MDx Bacterio-pulverisation in the BioRobot® MDx workstation in a final 100 µL volume (Qiagen GmbH, Hilden, Germany). Seven PCR primer pairs and seven probes were designed for the specific detection of *Bacillus anthracis* (anthrax), *Borrelia recurrentis* (louse-borne relapsing fever), *Bartonella quintana* (trench fever), *Rickettsia prowazekii* (epidemic typhus), *Salmonella enterica* Typhi (typhoid fever), *Poxvirus* (smallpox) and *Yersinia pestis* (plague) (Table 1). Real-time PCR amplification was performed using the QuantiTech Probe PCR Kit (Qiagen) and a 7900HT Fast Real-Time PCR System (Applied Biosystem, Courtaboeuf, France). Each well of a 384-well plate was filled with 10 µL Mix Quantitech, 2 µL sterile water, 2 µL of 2 pmol/µL probe, 0.5 µL forward primer (10 pmol/µL), 0.5 µL reverse primer (10 pmol/µL) and 5 µL DNA. Amplification consisted of 15-min activation at 95°C followed by 50 cycles of 30-sec denaturation at 95°C and 1-min hybridisation at 60°C. In every plate, two wells containing sterile water and two wells containing DNA extracted from dental pulp collected from skeletons devoid of anthropologic evidence of infection were used as negative controls.

Suicide PCR detection of *R. prowazekii*

The Douai specimens were further examined with a suicide nested PCR protocol after conventional phenol chloroform DNA extract [4]. The program PerlPrimer version 1.1.6 was used to design PCR primers. The first suicide nested-PCR targeted a 206-base pair fragment of the *Rickettsia prowazekii* DNA invertase Pin-like protein (ORF0698; gi|3861237|emb|AJ235273.1|) by combining external primers RpDet-F1 5′-GTTGGATATATAA-GGGTTTC-3′ and RpDet-R2 5′-CCGAGTCTATCTAATTT-CCA-3′ and internal primers RpDet-F3 5′-ATGATCGTCAAG-TGTTCGAT-3′ and RpDet-R4 5′-TAGACAGTCGCCATCT-TGTA-3′; the final expected PCR product was 152 bp (Table 2). This region had never been amplified in our laboratory. The first round PCR mix contained 1.6 µL $MgCl_2$, 0.5 µL bovine serum albumin (BSA), 2 µL Master Mix (Light Cycle® FastStart DNA Master Sybr Green, Roche Applied Science, France), 1 µL of a 10 µM solution of RpDet-F1, 1 µL of a 10 µM solution of RpDet-R2, 8.9 µL sterile water and 5 µL DNA (experimental tube) or 5 µL sterile water (negative control tube). The first round of amplification was done using a LightCycler™ apparatus (Roche Diagnostics) and the following conditions: 10-min activation at 95°C followed by 45 cycles of 15-sec denaturation at 95°C, 20-sec

Figure 3. Featuring multiple burials discovered in Douai.
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hybridisation at 55°C and 25-sec elongation at 72°C. PCR products were recovered by centrifugation of capillaries at 380 g for 2 minutes. The nested PCR was performed in the capillary containing 1.6 µL $MgCl_2$, 0.5 µL BSA, 2 µL Master Mix, 1 µL of 10 µM solution of RpDet-F3; 1 µL of 10 µM solution of RpDet-R4; 8.9 µL sterile water and 5 µL first round PCR product. The second amplification in the LightCycler™ apparatus used the following conditions: activation at 95°C for 10 minutes and 45 cycles of denaturation at 95°C for 15 seconds, hybridisation at 57°C for 20 seconds, elongation at 72°C for 25 seconds. Nested PCR products were detected on a 2% agarose gel (Invitrogen™, Paisley, Scotland) in the presence of molecular weight marker VI (Boehringer Mannheim, Germany).

A second suicide nested-PCR targeted a 187-base pair fragment of the *R. prowazekii* glutamine amidotransferase-like protein (rpr_ORF0700; gi|3861237|emb|AJ235273.1|) by combining

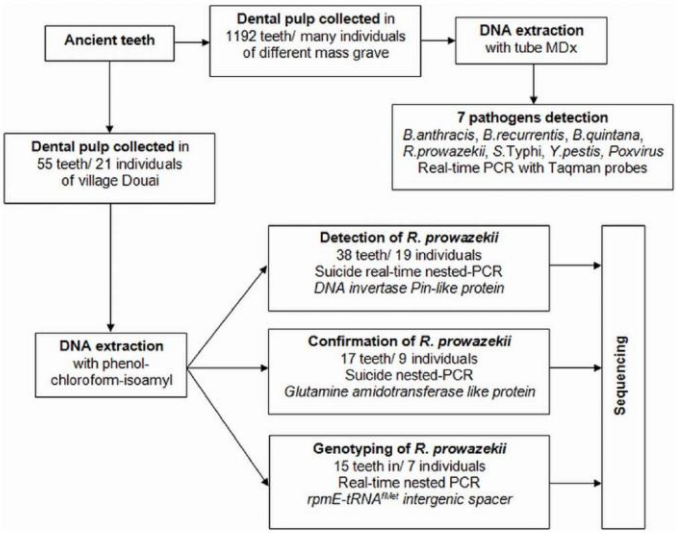

Figure 4. Summary of the materials and methods used in this study.
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Table 1. Primers for molecular detection of all pathogens into 1192 acient teeth.

Desired specificity	Gene	Name	Probe sonde and primers	Sequence
Bacillus anthracis (anthrax)	pag	Bant_pag_P	6 FAM- TAC CGC AAA TTC AAG AAA CAA CTG C -TAMRA	94 bp
		Bant_pag_F	5′- AGG CTC GAA CTG GAG TGA A -3′	
		Bant_pag_R	5′- CCG CCT TTC TAC CAG ATT T -3′	
Borrelia recurrentis (louse-borne relapsing fever)	unknown	Brec_P	6 FAM- CTG CTG CTC CTT TAA CCA CAG GAG CA -TAMRA	111 bp
		Brec_F	5′- TCA ACT GTT TTT CTT ATT GCC ACA -3′	
		Brec_R	5′- TCC TTA TGT TGG TTA TGG GAT TGA -3′	
Bartonella quintana (Trench fever)	ITS	Barto ITS_P	6 FAM- GCG CGC GCT TGA TAA GCG TG -TAMRA	102 bp
		Barto ITS_F	5′- GAT GCC GGG GAA GGT TTT C -3′	
		Barto ITS_R	5′- GCC TGG GAG GAC TTG AAC CT -3′	
Rickettsia prowazekii (typhus)	ompB	Rpr_ompB_P	6 FAM- CGG TGG TGT TAA TGC TGC GTT ACA ACA -TAMRA	134 bp
		Rpr_ompB_F	5′- AAT GCT CTT GCA GCT GGT TCT -3′	
		Rpr_ompB_R	5′- TCG AGT GCT AAT ATT TTT GAA GCA -3′	
Salmonella Typhi (typhoid fever)	unknown	Styp_put_P	6 FAM- GCT TTT TGT GAA GCA ACG CTG GCA -TAMRA	138 bp
		Styp_put_F	5′- CTC CAT GCT GCG ACC TCA AA -3′	
		Styp_put_R	5′- TTC ATC CTG GTC CGG TGT CT -3′	
Poxvirus (smallpox)	HA	Var_HA_P	6 FAM- AAG ATC ATA CAG TCA CAG ACA CTG T -TAMRA	100 bp
		Var_HA_F	5′- GAC KTC SGG ACC AAT TAC TA -3′	
		Var_HA_R	5′- TTG ATT TAG TAG TGA CAA TTT CA -3′	
Yersinia pestis (plague)	pla	Yper_PLA_P	6 FAM- TCC CGA AAG GAG TGC GGG TAA TAG G -TAMRA	98 bp
		Yper_PLA_F	5′- ATG GAG CTT ATA CCG GAA AC -3′	
		Yper_PLA_R	5′- GCG ATA CTG GCC TGC AAG -3′	

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external primers Rpro-F1 5′-ACTGTTATTACCGATCTTGCCA-3′ and Rpro-R1 5′-TGGTTGATGCTAGGTTATTTGG-3′ and internal primers Rpro-F11 5′-GTATTTAAGAATTTGATGCCACCA-3′and Rpro-R11 5′-GTTATTAGTCCAAATGACGTGAA-3′; the final expected PCR product was 130 bp (Table 2). This region had never been amplified in our laboratory. The first round of PCR was performed using a HotSartTaq DNA Polymerase Kit (Qiagen) with 0.8 µL $MgCl_2$, 0.2 µL HotStart Taq, 2.5 µL 10X PCR buffer, 2.5 µL dNTP, 0.5 µL BSA, 0.5 µL of a 10 µM solution of each Rpro-F1 and Rpro-R1, 12.5 µL sterile water and 5 µL DNA (experimental tube) or 5 µL sterile water (negative control tube). The first round of amplification was done in an ABI GeneAmp™ 2700 thermocycler (Applied Biosystems, CA, USA) under the following conditions: 10-min activation at

Table 2. Primers for detection and genotyping of *R. prowazekii* into ancient teeth of Douai.

	PCR	Name	Primers	Tm	Sequence zise	Reference
Detection of *R. prowazekii*	Real-time PCR	RpDet-F1 RpDet-R2	5′-GTTGGATATATAAGGGTTTC-3′ 5′-CCGAGTCTATCTAATTTCCA-3 ′	55°C	206 bp	rpr_ORF0698 site-specific recombinases, DNA invertase Pin-like protein
	Nested PCR	RpDet-F3 RpDet-R4	5′-ATGATCGTCAAGTGTTCGAT-3 ′ 5′-TAGACAGTCGCCATCTTGTA-3′	57°C	152 bp	
Confirmation of *R. prowazekii*	PCR standard	Rpro-F1 Rpro-R1	5′-ACTGTTATTACCGATCTTGCCA-3′ 5′-TGGTTGATGCTAGGTTATTTGG-3′	58°C	187 bp	rpr_ORF0700, glutamine amidotransferase-like protein
	Nested PCR	Rpro-F11 Rpro-R11	5′-GTATTAAGAATTTGATGCCACCA-3′ 5′-GTTATTAGTCCAAATGACGTGAA-3′	62°C	130 bp	
R. prowazekii genotyping	Real-time PCR	rpmE-F1 rpmE-R2	5′-CCGGAAATGTAGTAAATCAATC-3′ 5′-CTGAGAATTTAAAGATTTATCTG-3 ′	59°C	210 bp	Yong Zhu et al., 2005 rpmE-tRNA^{fMet} intergenic spacer
	Nested PCR	rpmE-F3 rpmE-R4	5′-CTTTCGATAGCAAGAAAGAAGC-3 ′ 5′-CAGAGTATTAGTAGACGATACG 3′	62°C	115 bp	

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95°C and 45 cycles of 30-sec denaturation at 95°C, 45-sec hybridisation at 58°C and 90-sec elongation at 72°C. The PCR products were purified using the Millipore plate protocol and suspended into 40 µL water. The second round of PCR was performed using 0.8 µL $MgCl_2$, 0.2 µL HotStart Taq, 2.5 µL 10X PCR buffer, 2.5 µL dNTP, 0.5 µL BSA, 0.5 µL Rpro-F11, 0.5 µL Rpro-R11, 12.5 µL sterile water and 5 µL first round PCR product. The second amplification was performed using the following conditions: 10-min activation at 95°C and 45 cycles of 30-sec denaturation at 95°C, 45-sec hybridisation at 62°C and 90-sec elongation at 72°C. Nested PCR products were detected on a 2% agarose gel (Invitrogen™, Paisley, Scotland) in the presence molecular weight marker VI (Boehringer Mannheim, Germany).

Nested PCR products were purified using the Millipore plate protocol and suspended in 50 µL water. The sequencing reaction was carried out in a tube containing 3 µL Big Dye Terminator, 0.5 µL forward or reverse primer, 3.5 µL purified PCR product and 3 µL sterile water using the following conditions: activation at 95°C for 5 minutes followed by 25 cycles consisting of 30-sec denaturation at 96°C, 20-sec hybridisation at 55°C, 4-min elongation at 60°C and 7-min extension at 15°C. The sequencing products were purified on Séphadex® G50 5% gel and analysed with the ABI PRISM 3100 Genetic Analyser (HITACHI). The sequences were read and corrected by using the software ChromasPro version 1.34 and then aligned with BLAST to compare the sequences available in GenBank.

Genotyping of R. prowazekii

The software PerlPrimer version 1.1.6 was used to design two pairs of primers targeting a 210-bp sequence (external primers: rpmE-F1 5′-CCGGAAATGTAGTAAATCAATC-3′ and rpmE-R2 5′-CTGAGAATTTAAAGATTTATCTG-3′) and a 115-bp sequence (internal primes: rpmE-F3 5′-CTTTCGATAGCAA-GAAAGAAGC-3′ and rpmE-R4 5′-CAGAGTATTAGTAGAC-GATACG 3′) on the rpmE-$tRNA^{fMet}$ intergenic spacer (gi|56967982|gb|AY695449.1|) (Yong Zhu et al., 2005) (Table 2). Genotyping was performed by mixing 1.6 µL $MgCl_2$, 0.5 µL BSA, 2 µL Master Mix, 1 µL of 10 µM solution of rpmE-F1; 1 µL of 10 µM solution of rpmE-R2, 8.9 µL sterile water and 5 µL DNA (experimental tube) or 5 µL sterile water (negative control tube) in a Stratagene plate. The first round of amplification was performed in a Stratagene™ thermocycler (Agilent Technologies Company) using the following conditions: activation at 95°C for 10 minutes, 45 cycles of denaturation at 95°C for 30 seconds, hybridisation at 59°C for 30 seconds, elongation at 72° C for 1 minute. Nested PCR was realised in a Stratagene plate containing 1.6 µL $MgCl_2$, 0.5 µL BSA, 2 µL Master Mix, 1 µL of 10 µM solution of rpmE-F3, 1 µL of 10 µM solution of rpmE-R4, 8.9 µL sterile water and 5 µL first round PCR product. The second amplification in the Stratagene™ thermocycler was performed using the following conditions: activation at 95°C for 10 minutes followed by 45 cycles consisting of denaturation at 95°C for 30 seconds, hybridisation at 62°C for 30 seconds and elongation at 72°C for 1 minute. The nested PCR products were detected on a 2% agarose gel (Invitrogen™) in the presence of molecular weight marker VI (Boehringer Mannheim). The PCR products were sequenced as described above.

Prevention of DNA contamination

all manipulations of ancient materials were done in two successive laboratories where *R. prowazekii* had never been previously amplified. Each step was conducted in a separate room under a hood with air-capture. All instruments were sterilised and used only once. No positive control was included, and one negative control consisting of sterile water was used for each three or five samples.

Results

High-throughput detection of pathogens

A total of 1.192 dental pulp specimens collected from several burial sites in France were analysed by high throughput detection, including 40 specimens collected in Douai tested blindly. Whereas the negative controls remained negative, high throughput real-time PCR detected *B. quintana* DNA in 1/40 dental pulp specimens, and no other pathogen was detected in these 40 specimens. We then attempted to detect *R. prowazekii* using a more sensitive technique.

Molecular detection of *R. prowazekii*

In all of the experiments, the negative controls remained negative. The first suicide real-time nested PCR detected *R. prowazekii* DNA in 2/38 (5.3%) ancient teeth collected from 2/19 (10.5%) different individuals (coded as A6-4 and A10-19). Sequence alignment yielded 100% sequence similarity with the reference *R. prowazekii* strain Madrid E (Genbank accession emb|AJ235273.1|RPXX04). As for the second suicide PCR, it detected *R. prowazekii* DNA in 1/17 (5.9%) dental pulp specimens collected from 1/9 (11%) different individuals (coded as 126/220-39) (Figure 5).

Genotyping of *R. prowazekii*

In the genotyping, the negative controls remained negative while we amplified the *R. prowazekii* rpmE/$tRNA^{fMet}$ intergenic spacer sequence in 3/15 (20%) ancient teeth collected from 3/7 (43%) different individuals (coded as 075-24, 077-27, 1074-35). All PCR products yielded an identical sequence exhibiting 100% sequence similarity to *R. prowazekii* type B, which is characterised by a T to C substitution at position 111 (Figure 6). Merging all of the PCR results yielded positive detection of *R. prowazekii* DNA in 6/55 (11%) teeth collected from 6/21 (28.6%) individuals.

Discussion

The results presented herein were interpreted as authentic. Indeed, we selected teeth that had a closed apex and that were free of dental caries and traumatic lesions to minimise any risk of external contamination of the dental pulp. For the first time, we used a high throughput paleomicrobiological approach to screen 1192 teeth for seven infectious agents (8,344 tests). This technique is less sensitive than suicide PCR but is well adapted to testing numerous samples. Our work was carried out in a laboratory where *R. prowazekii* had never been worked on, nor had *R. prowazekii* DNA been extracted. All operations were carried out under a hood with air-capture using sterilised instruments that were used only once. All the negative controls remained negative. We obtained an original sequence that was consistently found in several teeth collected in several individuals, its uniqueness thus giving much confidence. After blindly detecting *B. quintana* in one tooth from Douai, we decided to employ more sensitive techniques to recover *R. prowazekii* from other teeth from this site.

In this study, we observed that not all dental pulp specimens collected from a single individual yielded a positive PCR product. Such variability in the positivity of PCR-based detection has been previously observed in detecting *Yersinia pestis* DNA [4]. Specific *R. prowazekii* sequences were detected in 6/55 (11%) teeth collected in 6/21 (28.6%) individuals; this prevalence is significantly higher ($P<0.05$, test χ^2) than that previously reported in the Napoleon's

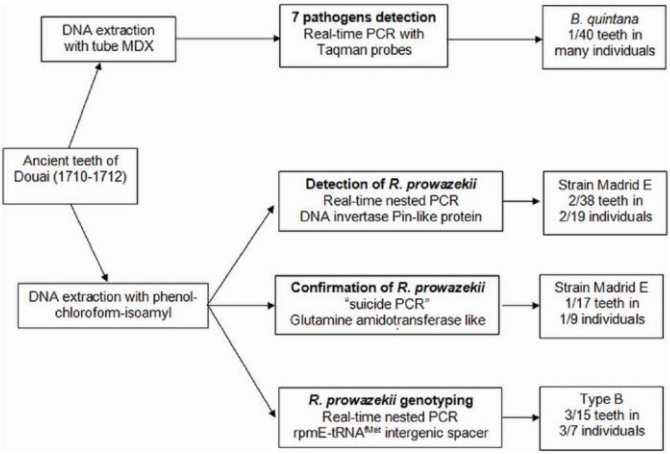

Figure 5. Summary of *R. prowazekii* detection and genotyping results.
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Grand Army study, which detected *R. prowazekii* in 4/72 (5.6%) teeth collected from 3/35 (8.6%) soldiers [15]. As we also found an individual infected by *B. quintana*, we suspect the circulation of louse-borne diseases in this place, but we did not recover *Borrelia recurrentis* DNA.

In the present study, we also took advantage of the dental pulp to detect and genotype 300-year-old *R. prowazekii* DNA. Dental pulp is a specialised conjunctive tissue occupying the central position in the tooth, where it is protected from the external environment by the dentine. We previously demonstrated that blood-borne bacteria could be detected in dental pulp both by culture and by molecular detection of specific DNA sequences [16–18]. In addition, dental pulp is the only conjunctive tissue that can persist for several thousand years after the degradation of other tissues because it is very well preserved within the dentine and enamel, which are the hardest tissues of the human body [4]. In 2004, Tran H Lam et al. showed that PCR amplifying a fragment of 286 base pairs of the 16S rRNA gene could identify several bacteria in the pulp of ancient teeth dated to the 17th century [19]. It is difficult to amplify fragments of DNA more than 300 base pairs from old samples [4,6]. For these reasons, we chose a fragment of 115–210 base pairs for amplification. Nested PCR, which is effected by two successive PCR reactions with two pairs of external-internal primers, can increase PCR's sensitivity and specificity considerably. With the combination of the benefits of real-time PCR [20] and nested PCR, we have successfully amplified an ancient bacterial DNA sequence from the 18th century and identified it as *R. prowazekii* genotype B. It was later shown that real-time PCR is a rapid, specific and sensitive method for detecting *R. prowazekii* in blood [20,21]. Sequence analysis of the rpmE/tRNA^{fMet} spacer in 15 modern *R. prowazekii* DNA samples found three genotypes: A, B and C. Genotype B had been sequenced in the avirulent *R. prowazekii* Madrid E strain (derived from a Spanish isolate [22]), in its virulent revertant *R. prowazekii* Evir in two blood isolates from Russia and Algeria, and in four lice collected in Rwanda and Burundi [23]. We herein demonstrated that this genotype was already present in 18th-century Europe.

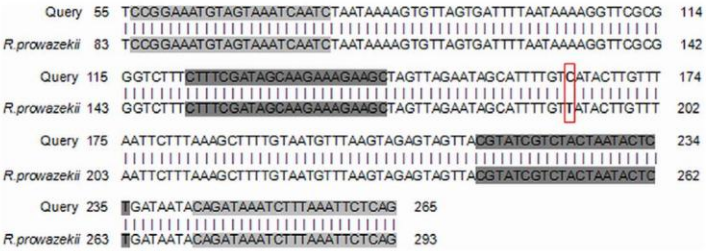

Figure 6. Result of Genbank alignments for genotyping. Grey boxes indicate the locations of PCR primers.
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Table 3. Dental pulp: a source for the paleomicrobiology of ancient epidemics.

Epidemic	Site - date	Materials and Methods	Results	Reference
Plague	Lambesc – 1590 and Marseille – 1722 (France)	Dental pulp, DNA amplification by PCR, gene RpoB (133-bp) and gene pla (300-bp)	*Y. pestis* detected in 6/12 teeth	[3]
	Saint-Côme and Saint-Damien (Montpellier) – 14th century (France)	Dental pulp, "suicide" PCR, gene pla (148-bp)	*Y. pestis* detected in 20/23 teeth	[5]
	Sens: 5th–6th century, Dreux: 12th–14th century and Monpellier – 1348 (France)	Dental pulp, DNA amplification by PCR, spacer YP	*Y. pestis* strain Orientalis detected in 7/11 individuals	[31]
	Aschheim – 6th century (Upper Bavaria)	Dental pulp, "suicide" PCR, gene pla (148-bp)	*Y. pestis* detected in 2/6 teeth	[32]
	Vienne: 7th–9th century, Martigues: 1720–1721 and Marseille – 1722	Dental pulp, suicide-nested PCR, gene glpD (191-bp)	*Y. pestis* strain Orientalis detected in 5/46 teeth	[33]
	Lambesc – 1590, Saint-Pierre: 1628–1632, Draguignan: 1649–1650, Martigues: 1720–1721, Berre l'Etang: 1720–1721, Marseille – 1722 (France)	Dental pulp and spongy bone, immuno-detection by RDT, F1 antigen	*Y. pestis* detected in 19/28 individuals	[34]
Typhoid fever	Athens: 430–426 BC (Greek)	Dental pulp, "suicide" PCR, gene osmC-clyA (322-bp) and gene narC (360-bp)	*S. enterica* Typhi detected in 3/3 teeth	[35]
Rocky Mountain spotted fever	Maryland – 1901 (USA)	Immunohistology detection	Detection of *R. rickettsii*	[22]
Cat-cratch disease	Compiègne – 16th century, Montbéliard – 14th century and Paris – 13th century	Dental pulp of cats, nested PCR, gene groEL (269 – bp) and gene Pap31 (164 – bp)	*B. henselae* detected in 3/135 teeth of cats	[36]
Trench fever	Roaix: 2100–2200 BC and Peyraoutes: 2230–1950 BC (France)	Dental pulp, nested PCR, gene hemin-binding protein-E (283-bp) and gene groEL (269-bp)	*B. quintana* detected in 1/12 teeth	[6]
Typhus and trench fever	Vilnius – 1812 (Lithuania)	Lice and dental pulp, DNA amplification by PCR, gene dnaA (141–279 bp) and gene hbpE (282–429 bp)	*R. prowazekii* detected in 4/72 teeth, *B. quintana* detected in 7/72 teeth and 3/5 lice	[7]
Typhus and trench fever	Douai: 1710–1712 (France)	Dental pulp, real-time PCR and "suicide PCR", gene ITS (102-bp) and gene DNA invertase Pin-like protein (152–206 bp), gene glutamine amidotransferase-like protein (130–187 bp), rpmE-tRNA^{fMet} intergenic spacer (115-210 bp)	*B. quintana* detected in 1/40 teeth and *R. prowazekii* strain Madrid E type B detected in 6/55 teeth	Present work

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Despite the fact that typhus epidemics has been depicted in historical sources for two millennia or so [24], only few demonstrations of *R. prowazekii* issued from ancient materials have been provided. One individual diagnosed with fatal epidemic typhus in Maryland in 1901 was demonstrated by using immunohistological detection ninety years later to have died of Rocky Mountain spotted fever caused by *Rickettsia rickettsii* [25]. In another report, sequence-based analysis of remains of Napoleon's Grand Army soldiers yielded molecular evidence for *R. prowazekii* and *B. quintana* in an estimated one-third of the individuals [26]. The data herein reported remind us that epidemic typhus, a disease now confined to relatively limited geographic areas, had a broad geographic range of prevalence only a few centuries ago, being one of the plagues reported in historical descriptions (Table 3) [27]. In the past, several typhus epidemics have been described in central Africa [28]. Today, cases of typhus are still described in Peru [29] and in industrial cities of Russia [30], Algeria [31] and France [32]. Further studies applying the techniques herein described to the remains of individuals in America, Europe and Africa may help to paint a clearer picture of the evolution and spread of epidemic typhus in connection with human history.

Taken together, the results reported herein show clearly that an epidemic disease transmitted by lice prevailed during the long siege of Douai. Diseases transmitted by body lice were probably extremely frequent in the past. When sanitary arrangements are degraded, lice are likely to quickly expand and the population of lice can increase by 10% per day [33] when the conditions of hygiene are met [29]. Under these conditions, it is easy to imagine a *B. quintana* epidemic persisting in Europe for an extremely long time. The oldest trace of infection of humans by this bacterium goes back 4.000 years [34]. Epidemic typhus appeared in Europe later. The majority of authors suggest that it was introduced by the Spanish returning from America [35]. The war in Douai was that of the Spanish succession, and one can propose that it was imported by Spanish soldiers. It is interesting therefore to observe that the genotype B found here is identical to that of a Spanish isolate from the beginning of the XXth century in Spain. The first descriptions go back to Fracastor, and early in the 16th century a number of epidemics compatible with the diagnosis of typhus were reported. However, at the beginning of the 18th century, the clinical individualisation of epidemic typhus, like that of trench fever of the trenches, was not yet carried out. The first definition of typhus was provided by Boissier de Sauvages in 1772 [36].

In conclusion, the molecular diagnosis of past epidemic infections related to the teeth made it possible to identify the first outbreak of epidemic typhus in the 18th century in the context of a pan-European Great War. Working together, molecular biologists, dentists and anthropologists have explored burials from catastrophes to identify the prevailing epidemics in past centuries.

Author Contributions

Conceived and designed the experiments: DR. Performed the experiments: TNH MS CR. Analyzed the data: GA DR MD. Contributed reagents/materials/analysis tools: MS GA DR CR. Wrote the paper: TNH GA DR.

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Chapitre V

Metagenomics study of dental pulp revealing a 300-year-old plague episode

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Chapitre V – Avant propos

La construction d'un palais des congrès à Issoudun (Indres), une ville du centre de la France a permis de mettre au jour un charnier d'environ 200 individus. Ce charnier a été daté d'une période du 17ème au 18ème siècle. D'après les données anthropologiques, 80% des individus sont des enfants de 1 à 9 ans et 20% des autres individus sont des adultes de moins de 30 ans. La cause du décès de ces individus n'était pas encore identifiée malgré plusieurs tentatives de détection moléculaire. Dans cette étude, l'analyse métagénomique de la pulpe dentaire par pyroséquençage massif a été appliquée. Après l'extraction de l'ADN de la pulpe dentaire de 5 dents d'Issoudun par protocole phénol-chloroforme, l'ADN a été quantifié. Compte tenu de l'insuffisance d'ADN, l'ADN total a été enrichi avant le pyroséquençage. L'alignement des séquences du métagénome contre les databases NCBI des protéines disponibles a montré 2 séquences d'une même région du plasmide pPCP1 de *Yersinia pestis*. Pour confirmer ces résultats métagénomiques, les amorces réamplifiant un fragment de 300-pb de la séquence de *Y. pestis* détectée dans le métagénome ont été désignées. L'alignement de la séquence amplifiée par « PCR suicide» contre les séquences de 4 génotypes connus de *Y. pestis* a montré 98% de similarité. Le système de PCR multiplex en temps réel a été également utilisé pour détecter *Y. pestis* et d'autres pathogènes y compris *Bacillus anthracis*, *Borrelia recurrentis*, *Bartonella quintana*, *Rickettsia prowazekii*, *Salmonella enteria* serovar Typhi et *Poxvirus*. Le séquençage a identifié seulement le gène *pla* de *Y. pestis* avec 100% de similarité. Pour le génotypage de *Y. pestis*, l'ADN a été extrait à partir de la pulpe dentaire des 25 dents collectées des 16 squelettes d'Issoudun. *Y. pestis* génotype Orientalis a été détecté par amplification du fragment du gène *glpD* et séquençage dans ces 5/16 squelettes. En conclusion, l'analyse

métagénomique par pyroséquençage massif est une nouvelle technique qui peut être appliquée dans les diagnostics paléomicrobiologiques pour détecter tous les pathogènes suspectés et inattendus à partir des échantillons anciens. Nous avons appelé cette nouvelle approche «paléométagénomique».

Metagenomics study of dental pulp revealing a 300-year-old plague episode

Running title: Paleometagenomics of ancient plague

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Keywords: ancient plague, detection, ancient teeth, dental pulp, high-throughput sequencing, multiplex real-time PCR

Word count abstract : 111 (<150)

Word count text: 2670 (<4000)

Abstract

The cause of multiple deaths dating from 17th-18th centuries, Issoudun, France remained unidentified despite intensive DNA-based investigations targeting several deadly pathogens including *Yersinia pestis*. High-throughput sequencing of pooled dental pulp DNA surprisingly yielded two sequences exhibiting best match with a *Y. pestis* hypothetical protein gene. This results was confirmed by suicide PCR-sequencing of this gene. Real-time PCR-sequencing further detected *Y. pestis pla* and *glp*D gene sequences in 5/16 individuals in Issoudun. This unexpected result prompted historical and anthropological reevaluation of the Issoudun burial site and identified different mass grave clusters related to different outbreaks. Paleometagenomics reported here for the first time, demonstrates its potential to identify unknown etiology of ancient outbreaks.

Introduction

An investigation by the Institut National de Recherches Archéologiques Préventives discovered 14 multiple graves in part of the ancient cemetery of Issoudun, France (Figure 1)(Briet, 2002; Castex et al., 2008). The minimum number of individuals was estimated at 203 (Castex *et al.*, 2008). Apart from one double burial, the graves contained 13-22 individuals deposed simultaneously and comprising of adults and a remarkable proportion (76%) of 1-14 year-old children (Figure 2). All the graves showed a very rational organisation of the deposits according to age criteria (Castex et al., 2008; Castex, 2008). Indeed, in most cases, adults and adolescents were the first occupants of the pit and they were distributed side-by-side and head-to-foot over three or four levels. Particularly, several immature individuals were mostly deposited on the belly of the adults or sometimes inserted against pit edges or into the remaining spaces (Figure 3). This type of disaster burial linked to sudden and massive mortality, can occur during war or an epidemic. The attention was therefore oriented to smallpox and measles epidemics because of the frequent affection in children and adolescents.

We launched a paleomicrobiology investigation of individuals collected from some graves with the objective to identify the pathogen in Issoudun (Drancourt and Raoult, 2005). However, molecular analyses were negative for targerted viruses as well as plague (Castex et al., 2008; Castex, 2008) and the cause of the multiple deaths at Issoudun remained unidentified.

In order to resolve the etiology of multiple deaths in Issoudun, we therefore developped a metagenomic approach by high-throughput sequencing of dental pulp DNA from additional individuals collected unexplored graves in Issoudun. Such deep sequencing of the dental pulp revealed the presence of *Yersinia pestis* DNA. This unanticipated result was

further confirmed by suicide-PCR amplification and real-time PCR amplification and sequencing of three different genes specific for *Y. pestis.*

Results

Metagenomic analysis

High-throughput pyrosequencing yielded 71,962 reads for a total of 20.4 million bp with an average 283-bp read length (range, 40-632 bp). Quality and duplicate filtering finally yielded 20,330 reads and Blastx on NR proteins-NCBI databases yielded a total of 3,594 hits. Two sequences (0.009% of the sequences) of 551-pb (GenBank accesssion number: JX026664.1 with 99% coverage and 99% identity) and 564-bp (GenBank accesssion number: EU 363768.1 with 99% coverage and 98% identity) exhibited an overlap of 448-bp and best-match hits with *Y. pestis* biovar Microtus hypothetical protein fragments of 102-amino-acid (GenBank accesssion number: NP 995570.1 with 55% coverage, 91% identity and 2e-62 E-value) and 117-amino-acid (GenBank accesssion number: NP 995570.1 with 62% coverage, 84% identity and 5e-63 E-value), respectively.

Suicide PCR-sequencing

Suicide PCR-sequencing yielded a 293-pb amplicon while all negative controls remained negative. This amplicon exhibited 98% sequence identity with *Y. pestis* Genbank accession NP 995570.1 and 96% sequence identity with the two sequences derived from high-throughput sequencing (Figure 5). This sequence was found in the pPCP1 plasmid of all *Y. pestis* strains (Genbank accession: gi|5763810|emb|AL109969.1| for Orientalis biovar, gi|320017637|gb|CP001611.1| for Medievalis biovar, gi|108782179|gb|CP000310.1| for Antiqua biovar and gi|45357364|gb|AE017046.1| for Microtus biovar).

Multiplex real-time PCR amplification

All negative controls remained negative. Among the seven tested pathogens, only *Y. pestis* was detected from the pooled DNA of five Isssoudun teeth. After the first real-time PCR positive for this pathogen with 31 Cycle threshold (Ct) value, the second also confirmed this result with 32 Ct value. Sequencing yielded 98-bp sequence of *Y. pestis pla* gene. BLAST alignment showed that ten first-results specifically matched *Y. pestis* pPCP1 plasmid with 182 total score, 100% coverage, 100% identity and 4e-43 E-value (Genbank accessions: HE576987.1, CP002958.1, CP002180.1, CP001611.1, HM807366.1, CP001596.1, CP001592.1, CP001588.1, CP000900.1, DQ772765.1).

Confirmation of Y. pestis detection

All negative controls remained negative. Real-time PCR detected *Y. pestis pla* gene in 2/25 dental pulp specimens collected from 2/16 Issoudun individuals. Sequencing yielded 98-bp with 100% sequence identity with *Y. pestis pla* gene. PCR-sequencing further identified a 144-bp *Y. pestis* glpD sequence exhibiting a 93-bp deletion characteristic of *Y. pestis* Orientalis biovar in 6/25 dental pulp specimens collected in 5/16 individuals. Overall, *Y. pestis* was detected in 5/16 individuals recovered from 3/5 multiple burials (Table 2). Two individuals 109-11 and 147-15 were positive for both *Y. pestis* pla and glpD detections and three individuals were positive for one of the two molecular detections. Interestingly, skeleton 147-16 positive for *glp*D gene detection, had been initially incorporated into pooled DNA used for paleometagenomics.

Discussion

The detection of *Y. pestis* DNA in the dental pulp specimens collected from the historical burial site at Issoudun was firmly assessed. Indeed, all negative controls introduced in every experiments remained negative

excluding in-laboratory contamination. In particular, reagents of GenomiPhi V2 DNA Amplification kit which may have been a source of false positive plasmid detection, remained negative. Data issued from a neutral metagenomic study were confirmed by targeted PCR-based studies. The identification of PCR products was ascertained by sequencing which showed best matches with *Y. pestis* specific reference sequences.

Starting from our first work done by PCR in 1998 (Drancourt 1998), we have chosen to use the dental pulp as a sample material, rather than the remains of bone, and we have since repeatedly used this technique (Raoult2000). It was recently confirmed by another team that the dental pulp had a superior richness of microbial ancient DNA compared to bone samples (Schuenemann2010; Bos2011). This topic had been the subject of controversy because of a single negative result (Gilbert2004) but multiple studies from different teams have confirmed that the use of the dental pulp PCR allowed to obtain satisfactory results and helped identifying epidemics including plague in five European countries (France, Germany, The Netherlands, England and Italy)(Haensch2010; Raoult2000; Bianucci2008; Bianucci2009; Drancourt1998; Drancourt2004; Drancourt2007; Tran2001b) and typhus in France and Lithuania (Nguyen2010; Raoult2006). Interestingly a recent study tested another amplification system which allowed to recover most of *Yersinia pestis* genome from medieval London plague specimens (Schuenemann2010; Bos2011).

In this study, we observed that high-throughput sequencing yielded 40-632-bp fragments. In particular, bacterial reads were of 196 +/- 114 bp and *Y. pestis* DNA fragments were of 551-564-bp. This observation contrasts with various theories based on mimicking modern experiments predicting that ancient DNA would be degraded into fragments of less than 100-bp (Lindahl, 1993; Poinar, 1996; Smith, 2001). Based on these theoretical data, it was predicted that the detection of larger fragments of ancient DNA

would not be possible and would necessarily result from contamination. The data reported here indicate that theories of ancient DNA fragmentation with time, especially old microbial DNA, are not consistent with experimental reality. Figure 6, showing the size of *Y. pestis* amplicons obtained by different teams throughout the world, shows that most researchers are able to amplify bacterial DNA fragments larger than 100-bp.

The fact that metagenomic analysis yielded *Y. pestis* sequences was unexpected because some specimens collected at Issoudun had been previously tested negative in our laboratory using the same real-time, multiplex PCR (Nguyen-Hieu et al., 2010). In particular, previous searches for *Y. pestis* also remained negative, leaving the Issoudun site without epidemic etiology. Previous investigations had been done using dental pulp extracted from teeth from children and teenagers; accordingly, such teeth exhibited an opened apex which may not ensure a good preservation of the ancient DNA. Also, *Y. pestis* infection of the dental pulp may differ in deciduous teeth from that we previously observed in adult, permanent teeth. In the present work, another series of teeth with closed apex collected from different, unexplored graves had been sent in our laboratory for investigation. The unexpected detection of plague prompted a reappraisal of the multiple burial site in Issoudun. Archives recorded five historical events that may have lead to mass grave in Issoudun following 1650: the "Fronde" war in 1650-1652, a putative smallpox or measles epidemics in 1658, famine in 1661-1662, acknowledged high mortality at the end of the reign of the "Sun King" Louis XIV in 1693-1694 and a long and hard winter in 1709 (Castex et al., 2008; Castex, 2008). These Issoudun graves dating from the late 17th to early 18th centuries, were aligned in relatively clear rows and in the same orientation, excepting two multiple burials S153 and S149 (Figure 4). These latters belonged to a posterior episode of massive mortality (Castex *et al.*, 2008). While specimens previously tested negative

in our laboratory had been recovered only from individuals buried in the first episode of massive mortality, thirty dental pulp specimens investigated in the present study were collected from both episodes. Molecular data therefore indicated that the Issoudun site was indeed comprised of several, different epidemic episodes including at least one episode of plague.

This study offers the proof-of-concept that metagenomics analyses are able to resolve the etiology of past epidemics. High-throughput sequencing for ancient plague was reported in two previous, twin-publications (Bos et al., 2011; Schuenemann et al., 2011). These works however enriched total DNA in targeted *Y. pestis*-DNA by using a *Y. pestis* DNA microarray before metagenomic analysis. At the opposite, we herein used a neutral, untargeted approach after whole DNA enrichment to achieve a sufficient total DNA concentration for pyrosequencing. We need to have a deep sequencing as in our case only 0.009% of sequences were usefull to identify the causative agent. The result showed that high-throughput sequencing had high sensibility for preliminary detection of expected and unexpected pathogens in the ancient dental pulp. We propose that high-throughput sequencing of total DNA could be used as a first-line technique for the discovery of pathogens in ancient specimens.

In conclusion, untargeted metagenomics by high-throughput sequencing is a suitable method for detecting pathogen from ancient specimens during paleomicrobiology investigations. It could be applied to orphan past infections in quest of etiology, such as the one known as suette or English sweating sickness which swept in the 15-16th centuries in Great-Britain then Europe (Thwaites et al., 1997). We propose to name this approach paleometagenomics.

Methods

Source of materials

Thirty dental pulp specimens collected as previously described (Nguyen-Hieu *et al.*, 2011) from sixteen Issoudun skeletons were used for metagenomic analysis and further PCR-based experiments (Figure 4). These skeletons were found in five multiple burials (S109, S119, S147, S152 and S153) at Issoudun (1693-1709).

DNA extraction

The dental pulp DNA was extracted using a modified phenol-chlorofom protocol. Briefly, each 1.5 mL Eppendorf tube containing dental pulp powder was added 10 µL proteinase K 25 mg/mL, 10 µL dodecylsulfate sodium 10% and 200 µL sterile water. The digestion was done with overnight incubation and agitation at 56°C. This digestion solution and 220 µL phenol (phenol/chloroform/isoamyl alcohol, 25:24:1 stabilized, BIOSOLVE, Valkenswaard, The Netherlands) were added into Phase Lock Gel Heavy tube (Phase Lock Gel Heavy, 5 PRIME GmbH, Hamburg, Germany) before centrifugation at 6,076 x g for 5 min. The upper phase was transfered to a new Phase Lock Gel Heavy tube and 220 µL phenol were added before 5-min centrifugation at 6,076 x g. The upper phase was then added into sterile Eppendorf tube containing 1 µL glycogen, 44 µL ammonium acetate 10M and 440 µL absolute ethanol. DNA was precipited by overnight incubation at -20°C. The supernatant was removed after 30-min centrifugation at 15,100 x g at 4°C and 440 µL ethanol 70% were added. After 30-min centrifugation at 15,100 x g at 4°C, the supernatant was removed and the pellet was dried at 56°C for 10-min. A 30 µL-volume TE buffer 1M was added before overnight dissolution at 4°C and storage at -20°C.

High-throughput sequencing

Total DNA extracted from five dental pulp specimens randomly collected from five Issoudun skeletons (109-17, 119-22, 147-16, 152-8 and 153-2) was pooled and then quantified using PicoGreen Quant-IT kit (Invitrogen,

Saint-Aubin, France) and the Tecan Genios Fluorometer. Because of the insufficience of DNA quantity (total 7.8 ng of the pooled DNA) for pyrosequencing (500 ng DNA required), 1µL pooled DNA was enriched using GenomiPhi V2 DNA Amplification Kit (GE Healthcare Life Sciences, Saclay, France). High-throughput sequencing of the pooled-enriched DNA was done using the GS-FLX genome sequencer (FLX Roche 454 LifeSciences) according to manufacturer's protocol. All obtained reads were filtered with 21 cut-off of quality and 65 minimal length by using CAMERA resource (Sun et al., 2011). Duplicated reads were then eliminated and Blastx on NR proteines-NCBI databases was performed with e-25 E-value and three alignment results from the first.

Suicide PCR-sequencing

A new primer set was designed for suicide PCR-amplification (Raoult et al., 2000) of the *Y. pestis* sequences identified by high-throughput sequencing. The PCR was performed using 0.8 µL $MgCl_2$, 2.5 µL 10X PCR buffer, 0.2 µL HotStart Taq (Qiagen), 2.5 µL dNTP, 0.5 µL bovine serum albumin (BSA), 0.5 µL forward primer Yp-F: 5'-GTCGTGTCTTACCGGGTTG-3', 0.5 µL reverse primer Yp-R: 5'-CGACGCTCAAGTCAGAGGT-3', 12.5 µL sterile water and 5 µL of pooled DNA. Negative controls including reaction mix plus sterile water or reagents of GenomiPhi V2 DNA Amplification Kit were added. The amplification was performed using the following conditions: 15-min activation at 95°C, 45 cyles of 40-sec denaturation at 94°C, 40-sec hybridization at 60°C and 40-sec of elongation at 72°C, and 7-min of extension at 72°C. PCR products were purified according to protocol of QIAquick PCR Purification Kit and suspended in 50 µL EB buffer (Qiagen). The sequencing reaction was carried-out in a tube containing 3 mL Big-Dye Terminator (Applied Biosystems, CA, USA), 0.5 mL forward or reverse primers, 6.5 mL purified PCR product using the following conditions: 5-min activation at 95°C followed by 25

cycles consisting of 30-sec denaturation at 96°C, 20-sec hybridization at 55°C, 4-min elongation at 60°C and 7-min extension at 15°C. The sequencing products were purified on Sephadex G50 5% gel and analysed with the ABI PRISM 3100 Genetic Analyser (Applied Biosystem, Courtaboeuf, France). The sequences were read and corrected by using the software ChromasPro version 1.34. Finally, the multiple sequence alignment was performed using Clustal Omega program via EMBL-EBI to compare *Y. pestis* sequences.

Multiplex real-time PCR amplification

The pooled DNA were used for multiplex PCR-detection of seven pathogens including *Bacillus anthracis, Borrelia recurrentis, Bartonella* sp., *R. prowazekii, Salmonella enterica* Typhi, *Poxvirus* and *Y. pestis* as previously described (Tran et al., 2011b) (Table 1). Real-time PCR amplification was performed using the QuantiTech Probe PCR Kit (Qiagen) and a 7900HT Fast Real-time PCR System (Applied Biosystem, Courtaboeuf, France). Several negative controls including reaction mix plus sterile water were added. All positive results were separately confirmed again and then controlled by sequencing and BLAST alignment with the sequences available in Genbank.

Confirmation of Y. pestis detection

Total DNA extracted from 25 dental pulp specimens collected in 16 individuals was used for real-time PCR and sequencing of *Y. pestis pla* and *glp*D genes. Briefly, real-time PCR amplification of *Y. pestis pla* gene was performed in a CFX96TM Real-Time System (Bio-Rad, Singapore) with 10 µL reaction mixture Quantitech (Qiagen), 3.5 µL sterile water, 0.5 µL Taqman probe Yper-PLA-P (Table 1), 0.5 µL forward primer Yper-PLA-F, 0.5 µL reverse primer Yper-PLA-R and 5 µL DNA by using the following conditions: 15-min activation at 95°C, 50 cycles of 30-sec denaturation at 95°C and 1-min hybridization at 60°C and 30-sec extension at 45°C. The

PCR amplification of *Y. pestis glp*D gene (Drancourt et al., 2007) was performed with 0.8 µL $MgCl_2$, 2.5 µL 10X PCR buffer, 0.2 µL HotStart Taq (Qiagen), 2.5 µL dNTP, 0.5 µL BSA, 0.5 µL forward primer glpD-F3: 5'-CGCTGTTTCGAACATTCAGA-3', 0.5 µL reverse primer glpD-R3: 5'-GGCCAAGGCTTCACTTACCA-3', 12.5 µL sterile water and 5 µL DNA by using the following conditions: 15-min activation at 95°C, 50 cyles of 40-sec denaturation at 94°C, 40-sec hybridization at 58°C and 40-sec of elongation at 72°C and 7-min of extension at 72°C. Negative controls including reaction mix plus sterile water and DNA extracted from ancient dental pulp specimens previously confirmed by *Y. pestis*-DNA free detection were added. All PCR-positive results were controlled by sequencing and BLAST alignment with the sequences available in Genbank.

Prevention of DNA contamination

All manipulations of ancient materials were performed in separate rooms under a hood with air-capture. No *Y. pestis* was diagnosed or worked in parallel in our laboratory during these experiments. All disposable instruments were sterilised and used only once. No positive control was included in any PCR-based experiments.

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Figure 1: General view of the multiple burials at Issoudun.

Figure 2: Mortality profiles of immature individuals and young adults at Issoudun (Castex, 2008).

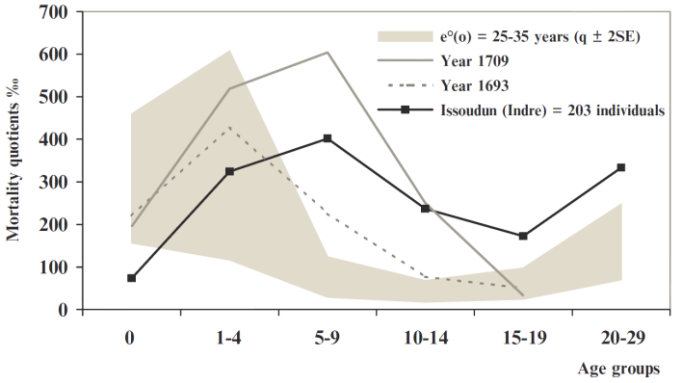

Figure 3: Multiple burials S119 containing 22 individuals and reconstitution of the organisation of the deposits (Castex, 2008).

Figure 4: Source of Issoudun materials with sex and age informations of each individual. M: male; F: female; ?: non-determinated sex, red colour: the first episode of massive mortality (late 17th – early 18th centuries); blue colour: the second episode of massive mortality.

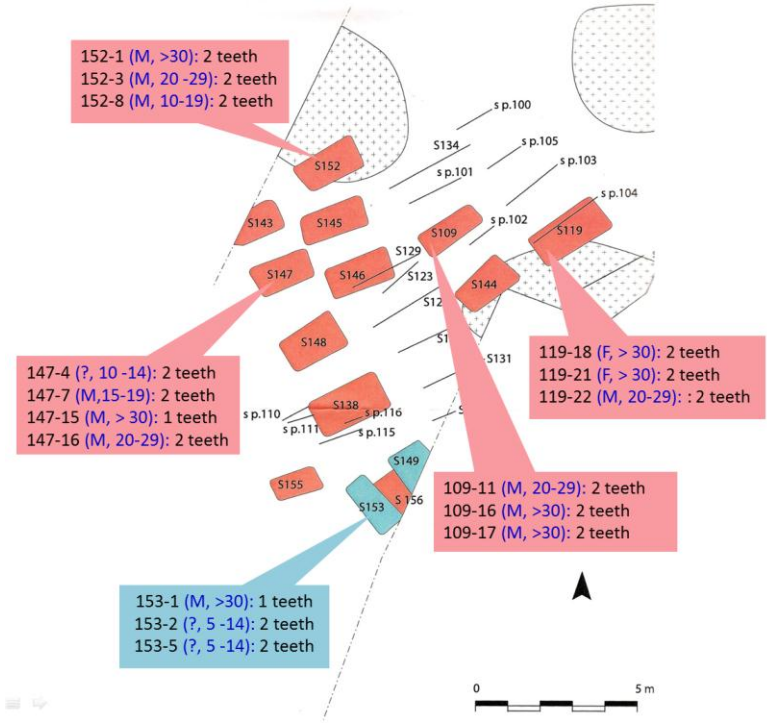

Figure 5: Multiple sequence alignment to compare the PCR-amplified sequence with *Y. pestis* sequences and pyrosequencing-detected sequence. Yp-Orientalis: *Y. pestis* Orientalis biovar, Yp-Medievalis: *Y. pestis* Medievalis biovar, Yp-Antiqua: *Y. pestis* Antiqua biovar, Yp-Microtus : *Y. pestis* Microtus biovar, G4Y6VJX04D48VY : partial sequence identified by high-throughput sequencing, Contig-PCR: sequence re-amplified by PCR-sequencing.

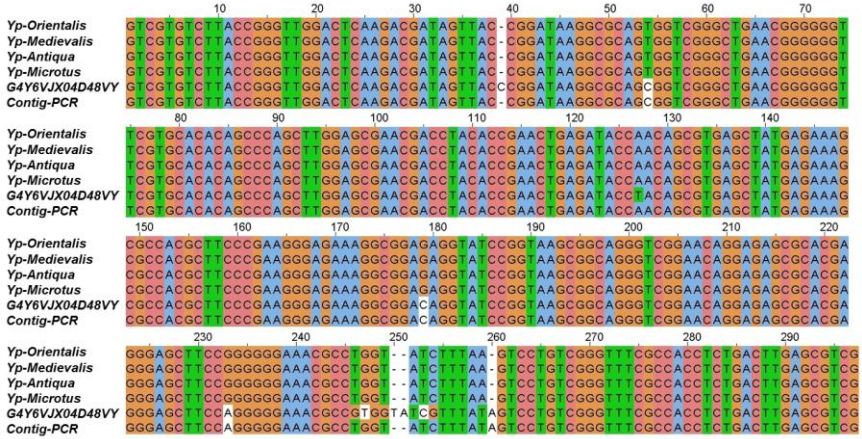

Figure 6: Length of microbial DNA fragments found by high-throughput pyrosequencing reads and PCR amplification of *Yersinia pestis* -specific sequences in ancient human specimens. M, metagenomics; PCR, polymerase chain reaction; RT-PCR, real-time PCR. PW, present work. Blue bars indicate successful detection; red bars indicate failed detection.

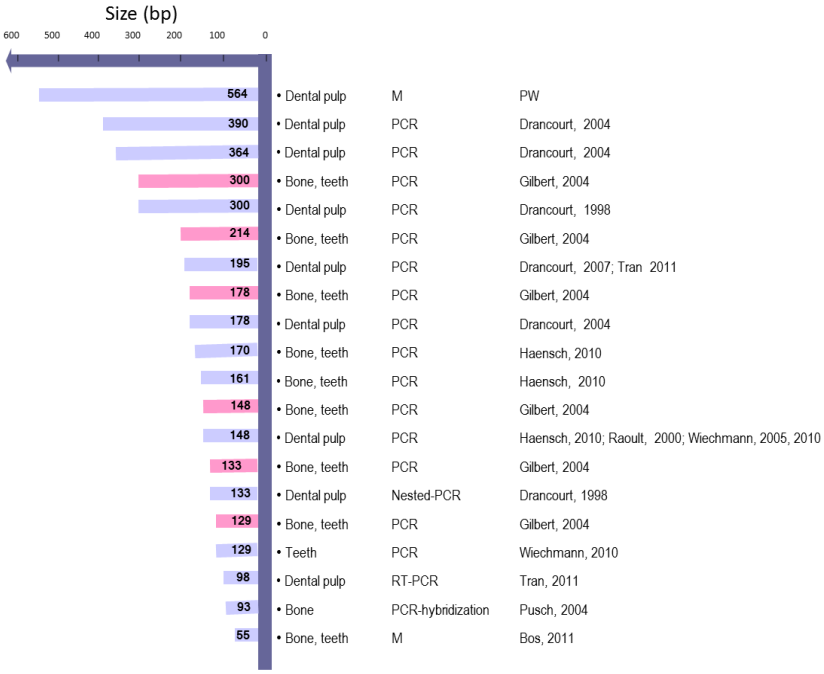

Figure 7: Bacterial DNA PCR-amplified from ancient specimens

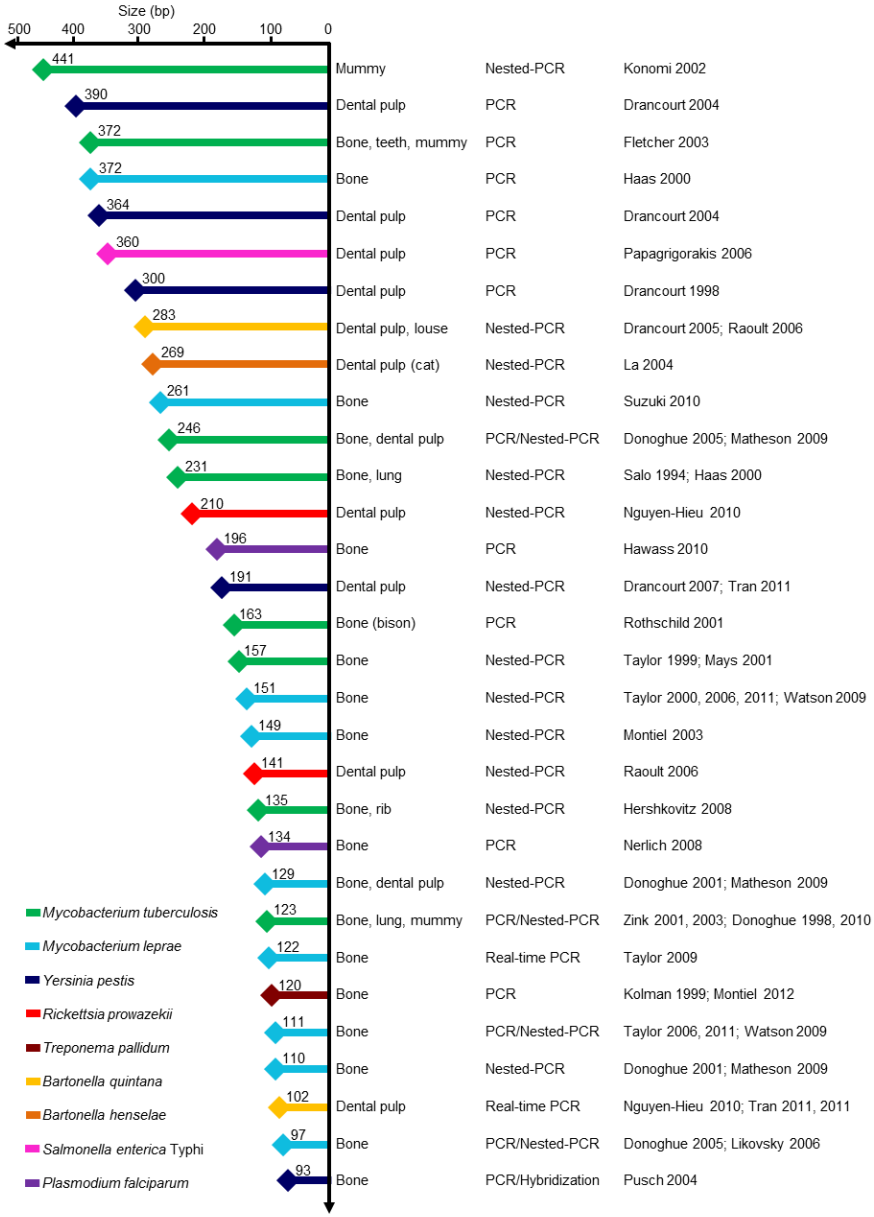

Figure 8: Metagenomic analyses of ancient specimens

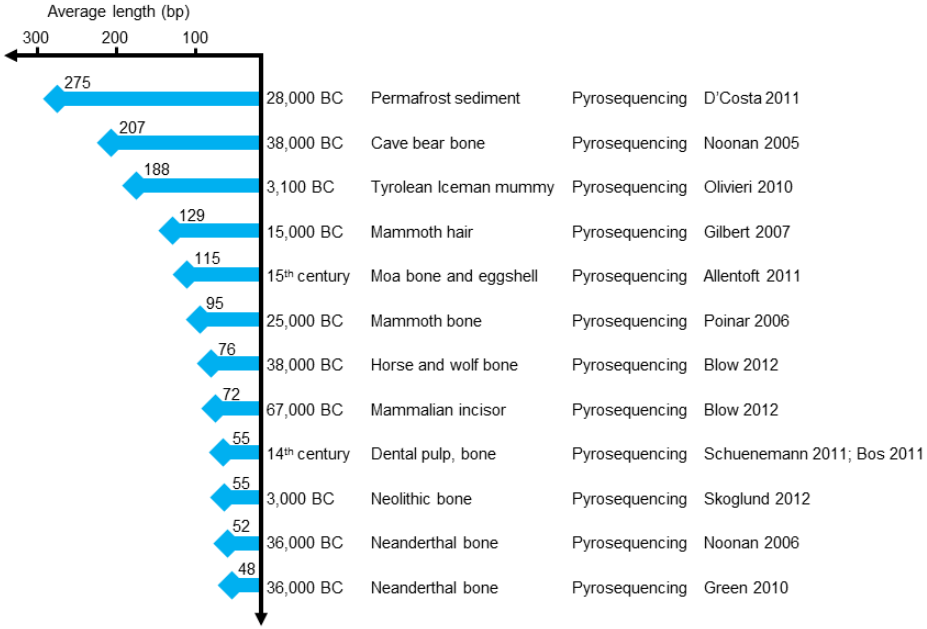

Table 1: Primers and probes for multiplex real-time PCR amplification (Tran et al., 2011b).

Targets	**Genes**	**Primers/ probes**	**Sequences (5'- 3')**	**Size**
B. anthracis	pag	Bant-pag-P Bant-pag-F Bant-pag-R	6 FAM- TACCGCAAATTCAAGAAACAACTGC -TAMRA AGGCTCGAACTGGAGTGAA CCGCCTTTCTACCAGATTT	94-bp
B. recurrentis	-	Brec-P Brec-F Brec-R	6 FAM- CTGCTGCTCCTTTAACCACAGGAGCA -TAMRA TCAACTGTTTTTCTTATTGCCACA TCCTTATGTTGGTTATGGGATTGA	111-bp
Bartonella sp.	ITS	Barto-ITS-P Barto-ITS-F Barto-ITS-R	6 FAM- GCGCGCGCTTGATAAGCGTG -TAMRA GATGCCGGGGAAGGTTTTC GCCTGGGAGGACTTGAACCT	102-bp
R. prowazekii	ompB	Rpr-ompB-P Rpr-ompB-F Rpr-ompB-R	6 FAM- CGGTGGTGTTAATGCTGCGTTACAACA -TAMRA AATGCTCTTGCAGCTGGTTCT TCGAGTGCTAATATTTTTGAAGCA	134-bp
S. enterica Typhi	-	Styp-put-P Styp-put-F Styp-put-R	6 FAM- GCTTTTTGTGAAGCAACGCTGGCA -TAMRA CTCCATGCTGCGACCTCAAA TTCATCCTGGTCCGGTGTCT	138-bp
Poxvirus	HA	Var-HA-P Var-HA-F Var-HA-R	6 FAM- AAGATCATACAGTCACAGACACTGT -TAMRA GACKTCSGGACCAATTACTA TTGATTTAGTAGTGACAATTTCA	100-bp
Y. pestis	pla	Yper-PLA-P Yper-PLA-F Yper-PLA-R	6 FAM- TCCCGAAAGGAGTGCGGGTAATAGG -TAMRA ATGGAGCTTATACCGGAAAC GCGATACTGGCCTGCAAG	98-bp

Table 2: PCR-based detection of *Y. pestis* DNA in the dental pulp of Issoudun teeth.

Burials	Skeletons	Teeth	*pla* gene	*glp*D gene
S109	109-11	1	+	-
		2	-	+
	109-16	3	-	+
		4	-	-
	109-17*	5	-	-
S119	119-18	6	-	-
		7	-	-
	119-21	8	-	-
		9	-	-
	119-22*	10	-	-
S147	147-4	11	-	-
		12	-	-
	147-7	13	-	-
		14	-	-
	147-15	15	+	+
	147-16*	16	-	+
S152	152-1	17	-	-
		18	-	-
	152-3	19	-	+
		20	-	+
	152-8*	21	-	-
S153	153-1	22	-	-
	153-2*	23	-	-
	153-5	24	-	-
		25	-	-

*: Dental pulps used for metagenomic analysis, +: PCR-sequencing-positive detection, -: PCR-negative detection

Supporting informations

References of Figure 7: Bacterial DNA PCR-amplified from ancient specimens [1-39].

References of Figure 8: metagenomic analyses of ancient specimens [40-51].

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CONCLUSIONS & PERSPECTIVES

Après avoir revu les données disponibles sur la microbiologie de la pulpe dentaire, notre premier travail expérimental sur la dégradation de l'ADN a mis en évidence que l'ADN des mycobactéries était plus résistant que l'ADN eucaryote. L'application de ces résultats dans la paléomicrobiologie suggère que les méthodes moléculaires basées sur les techniques de PCR peuvent détecter des fragments plus longs d'ADN bactérien à partir des échantillons anciens. Cette conclusion est en concordance avec les résultats de certaines études qui ont amplifié les fragments de plus de 350-pb d'ADN de *M. tuberculosis* et *M. leprae* à partir des échantillons anciens (Fletcher et al. 2003, Haas et al. 2000, Konomi et al. 2002).

En 2006, une première étude a identifié l'ADN de *B. quintana* et *R. prowazekii* à partir de la pulpe dentaire collectée des squelettes de soldats dans la Grande Armée de Napoléon. Cette détection a montré qu'un tiers des soldats ont été tués à Vilnius par l'épidémie du typhus. Cette épidémie constitue probablement une raison majeure à la défaite de la campagne de Napoléon en Russie, en 1812 (Raoult et al. 2006). Dans le deuxième travail de cette Thèse, un système de détection rapide de 7 pathogènes par PCR multiplex en temps réel, a été développé et appliqué pour détecter *B. quintana* et *R. prowazekii* souche Madrid E génotype B à partir de la pulpe dentaire ancienne des soldats morts à Douai pendant la guerre de la succession d'Espagne, 1710 – 1712. C'est la plus ancienne détection de typhus. Cette étude supporte l'hypothèse que le typhus a été introduit en Europe par les soldats espagnols au retour des conquêtes en Amérique.

Dans un troisième travail, l'analyse métagénomique par pyroséquençage massif a été utilisée pour détecter les microorganismes dans la pulpe dentaire des dents anciennes collectées du charnier d'Issoudun. La majorité des individus trouvés dans ce charnier datant du 17ème au 18ème siècle, sont des enfants dont la cause du décès n'était pas encore déterminée (Briet

2002, Castex et al. 2008, Castex 2008). L'identification des séquences de *Y. pestis* dans le métagénome a montré que le pyroséquençage était une technique sensible qui peut être utilisée en première ligne pour détecter tous les pathogènes suspectés et autres dans les échantillons anciens. Les résultats métagénomiques doivent être confirmés ultérieurement par les différentes techniques de PCR qui amplifient des gènes spécifiques des pathogènes identifiés du métagénome.

En conclusion, la pulpe dentaire ancienne est une source utile pour les diagnostics paléomicrobiologiques. Les deux études publiées en 2011 dans les «Proceedings of the National Academy of Sciences U.S.A» et dans «Nature», qui ont reconstruit le génome de *Y. pestis* ancien à partir de la pulpe dentaire ancienne (Bos et al. 2011, Schuenemann et al. 2011), ont conclu définitivement sur la question d'opportunité à d'utiliser la pulpe dentaire pour la recherche des pestes anciennes (Drancourt 2012a, Gilbert et al. 2004). Bien que *Y. pestis* génotype Orientalis a été proposée comme l'agent infectieux des deux grandes épidémies de peste dans le passé (Drancourt et al. 2004, Drancourt et al. 2007, Tran et al. 2011d, Tran et al. 2011b), les conclusions sont actuellement contradictoires (Haensch et al. 2010, Tran et al. 2011b, Wiechmann et al. 2010). Les futures études doivent se concentrer sur la recherche des génotypes de *Y. pestis* en relation avec les différents vecteurs entraînant l'explosion des épidémies (Drancourt & Raoult 2011, Drancourt 2012b, Drancourt 2012a). Le pyroséquençage massif utilisé en première ligne pour détecter tous les microorganismes dans la pulpe dentaire ancienne d'une façon très sensible et la confirmation des résultats métagénomiques par les PCRs ayant une haute spécificité sont suggérés comme une stratégie convenable aux diagnostics paléomicrobiologiques. Les méthodes moléculaires peuvent détecter les fragments plus larges d'ADN bactérien dans les échantillons anciens.

Pourtant, les techniques basées sur l'analyse de biomolécules non-ADN comme l'immunodétection, l'immuno-PCR, la spectrométrie de masse peuvent aussi être des outils complémentaires intéressants pour l'analyse de l'ADN ancien (Tran et al. 2011a).

Les résultats de ce travail de Thèse contribuent aux connaissances en paléomicrobiologie et proposent des nouvelles méthodes et stratégies de recherches paléomicrobiologiques.

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